An early effect of retinoic acid: Cloning of an mRNA (*Era-1*) exhibiting rapid and protein synthesis-independent induction during teratocarcinoma stem cell differentiation

(F9 stem cells/retinoids/gene expression/cDNA cloning)

GREGORY J. LAROSA* AND LORRAINE J. GUDAS†‡§

*Program in Cellular and Developmental Biology and †Department of Pharmacology, Harvard Medical School, Boston, MA 02115; and ‡Dana–Farber Cancer Institute, Boston, MA 02115

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ABSTRACT Vitamin A and its derivatives (retinoids) exhibit profound effects on the proliferation and differentiation of many cell types. However, the molecular mechanism by which retinoids exert these effects is unknown. Cultured murine F9 teratocarcinoma stem cells, which differentiate into nontumorigenic endoderm cells in response to retinoic acid (RA), have been used to identify genes regulated by RA. A cDNA library synthesized from F9 cells treated with RA for 8 hr has been screened with a cDNA probe enriched for sequences rapidly induced by RA, and a gene that exhibits the characteristics of a primary target for RA has been identified. This gene, early retinoic acid-1 (*Era-1*), encodes a 2.2- to 2.4-kilobase polyadenylated RNA; the level of *Era-1* mRNA rapidly and transiently increases up to 35-fold, depending on the concentration of exogenous RA. The increase in *Era-1* mRNA is dependent on the continuous presence of exogenous RA. The RA-associated increase in *Era-1* mRNA is seen even in the presence of protein synthesis inhibitors, but the increase is prevented by inhibitors of RNA synthesis such as actinomycin D. This increase in the steady-state level of *Era-1* mRNA in F9 cells is a very early effect of retinoic acid on gene expression in this differentiation system.

Retinoids, a class of compounds including retinol (vitamin A) and its natural (e.g., retinaldehyde and retinoic acid) and synthetic analogues, exert striking effects on cell proliferation, differentiation, and pattern formation during development (for reviews, see refs. 1 and 2). For instance, retinoic acid can mimic the action of the influential group of cells, the zone of polarizing activity, in the developing chick limb bud (3, 4), and retinoids cause specific alterations in the proximodistal pattern of regenerating amphibian limbs (5). In addition, retinoids can suppress the process of carcinogenesis *in vivo* (6) and the development of the malignant phenotype *in vitro* (2). The molecular mechanism(s) by which retinoids exert such potent effects on the growth, differentiation, and neoplastic transformation of a variety of different cell types have not been elucidated, even though many of these effects of retinoids have been known for over 60 years.

Retinoids induce differentiation and consequently modify gene expression in several types of cultured cells. Specific cellular genes induced by retinoids have been identified and cloned from differentiating HL-60 promyelocytic leukemia cells (7), cultured human epidermal keratinocytes (8), and murine teratocarcinoma cells (9–18). However, the relatively slow time courses of induction suggest that the genes that have previously been shown to be retinoid-inducible (7–18) do not represent the initial cellular response(s) to the

retinoids. For example, murine F9 teratocarcinoma stem cells, which differentiate in monolayer culture into parietal endoderm in response to retinoic acid (RA) (19), do not begin to express higher levels of laminin B1, collagen IV ($\alpha 1$), or other parietal endoderm-specific mRNAs until 18–24 hr after RA addition (10, 11, 13). Moreover, the induction of collagen IV ($\alpha 1$) and laminin B1 mRNAs by retinoic acid in F9 teratocarcinoma cells is prevented by inhibitors of protein synthesis such as cycloheximide (20), suggesting that prior production of protein intermediate(s) is required for the induction of these genes.

To delineate the initial responses of F9 teratocarcinoma cells to RA, we have attempted to isolate genes that exhibit properties characteristic of a primary response to RA; such properties would include the rapid induction of the specific mRNA following RA addition, the dependence of the induction on the continuous presence of RA, and the complete induction of the specific mRNA by RA in the absence of protein synthesis. We have used a procedure in which cDNA was enriched for RA-induced sequences by subtractive hybridization (21) and used as a probe to isolate cDNA clones from a cDNA library of RA-treated (8 hr) F9 cells. With this procedure we have isolated the cDNA for a gene, early retinoic acid 1 (*Era-1*), which exhibits properties of a primary response to RA. The increase in *Era-1* mRNA in response to RA represents a very early effect of RA at the RNA level in this system in which retinoids influence differentiation.

MATERIALS AND METHODS

Cell Culture and Nucleic Acid Isolation. F9 teratocarcinoma cells were cultured as described (13). Total cellular RNA was isolated by the guanidine hydrochloride method essentially as described (13, 22, 23). Poly(A)⁺ RNA was purified from total RNA by poly(U)-Sephadex column chromatography (24). Genomic DNA was isolated as previously described (25).

cDNA Library Construction and Screening. Poly(A)⁺ RNA was isolated from F9 teratocarcinoma cells treated for 8 hr with RA/cAMP/T [1 μ M RA/500 μ M dibutyryl cAMP/500 μ M theophylline (all from Sigma)]. Using 12 μ g of this poly(A)⁺ RNA, a cDNA library was constructed in the vector λ gt10 with modifications of the Gubler and Hoffman procedure (26), similar to those that have since been published (27, 28). The initial ligation and packaging of a portion of the cDNA resulted in a library of $\approx 5 \times 10^5$ independent recombinants. Approximately 40,000 plaques of this cDNA

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Abbreviations: RA, all-*trans*-retinoic acid; *Era-1*, early retinoic acid-induced gene 1; RA/cAMP/T, all-*trans* retinoic acid plus dibutyryl cAMP and theophylline.

§To whom reprint requests should be addressed.

library were screened using a "subtracted" cDNA probe; this probe had been enriched for sequences induced within 12 hr of RA treatment by two rounds of hybridization of RA/cAMP/T 12-hr cDNA with a 5-fold mass excess of poly(A)⁺ RNA from F9 cells treated for 12 hr with 500 μ M dibutyryl cAMP/500 μ M theophylline, followed by hydroxylapatite chromatography (21, 29). The resulting single-stranded cDNA was used to screen the library. Duplicate nitrocellulose filter replicas (30) were prehybridized and hybridized at 42°C in 50% (vol/vol) formamide/5 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl/0.015 M sodium citrate)/50 mM NaH₂PO₄/Na₂HPO₄ (pH 7.4)/5 mM EDTA/0.08% each Ficoll and polyvinylpyrrolidone/0.1% NaDodSO₄/200 μ g of denatured salmon sperm DNA per ml/50 μ g of poly(A) per ml/10% (wt/vol) dextran sulfate. Filters were washed in 0.5 $\times$ SSC/0.2% sodium pyrophosphate/0.1% NaDodSO₄ at 55°C. Plaques showing hybridization with the subtracted probe were rescreened using the same subtracted cDNA probe, and DNA was isolated from phage that still showed positive hybridization. For a third screen, the cDNA insert was isolated and labeled by the random primer/low-melting point agarose procedure (31) and hybridized to RNA blots of F9 RNA isolated at various times after RA addition. Initially, a 1.8-kb cDNA Era-1-encoding insert that hybridized to a rapidly induced RNA was isolated from the RA/cAMP/T 8-hr cDNA library. This 1.8-kb cDNA insert was used to rescreen 160,000 plaques of the library, and seven additional Era-1-containing clones were isolated, including a phage containing a 2.2-kb Era-1 cDNA. The phage was subsequently plaque-purified, and the 2.2-kb Era-1 cDNA insert was subcloned into pUC9.

Blot Hybridization Analysis. Genomic DNA was analyzed by the procedure of Southern (32). For RNA blot analysis, total RNA (2 μ g) was fractionated by electrophoresis in 1.0% agarose/2.2 M formaldehyde slab gels, stained with ethidium bromide, transferred to nitrocellulose filter membranes by blotting, and attached to the filters by baking at 80°C *in vacuo*. All filters were hybridized in the same solution as described for the library screening. The cDNA probes for the blot hybridizations were specific cDNA inserts, isolated and labeled with ³²P-labeled dCTP by the low-melting agarose/random-primer procedure (31). After a 16-hr hybridization, the RNA blots were washed in 0.2 $\times$ SSC/0.1% NaDodSO₄ at 60°C, and then exposed to Kodak XAR-5 film at -70°C. The degree of hybridization was quantitated by scanning densitometry of several exposures of each hybridization using an Helena Laboratories (Beaumont, TX) densitometer equipped with an automatic integrator.

RESULTS

Era-1 mRNA Expression During Teratocarcinoma Stem Cell Differentiation. The recombinant plaque that produced the best signal with the subtracted cDNA probe in the initial screening of the RA/cAMP/T 8-hr cDNA library was named Era-1, for early RA gene 1. After a positive signal was obtained in the second screening, the Era-1 cDNA insert was used to analyze the expression of the Era-1 gene during the differentiation of F9 teratocarcinoma stem cells in response to RA.

The Era-1 cDNA hybridizes to a 2.2- to 2.4-kb RNA that exhibits a 2- to 3-fold increase in steady-state level within 2 hr after RA addition to F9 teratocarcinoma stem cells, as assayed by RNA blot analysis (Fig. 1A). In contrast, laminin B1 mRNA level does not increase significantly until 24–48 hr after RA addition (Fig. 1A). When a high RA concentration (1 μ M) is added exogenously, the Era-1 mRNA level increases 25- to 30-fold over a 48-hr period (Fig. 1A), followed by a decline at 96 hr after RA addition (data not shown). The duration of this increase in mRNA level is dependent on the

RA concentration. If a lower concentration of RA (10 nM) is added exogenously, the Era-1 mRNA level increases 15-fold by 12–24 hr, followed by a 50% decrease in level between 24–48 hr (Fig. 1B). The more rapid decline in Era-1 mRNA after addition of 10 nM RA as compared with 1 μ M RA may be due to the conversion of RA to less active metabolites (34).

When F9 cells are grown for 8 hr in medium containing 1 μ M RA and then placed in medium without RA, a decrease in the Era-1 mRNA level results (Fig. 1C). This suggests that RA must be present continuously for the increase in the level of the Era-1 mRNA to be maintained. This result is in striking contrast to the level of laminin B1 mRNA, which does not decrease upon the removal of exogenous RA at 8 hr. In fact, the laminin B1 mRNA level continues to increase for at least 40 hr after the removal of RA (Fig. 1C).

We have previously shown that dibutyryl cAMP greatly enhances RA-induced expression of laminin B1 and collagen IV (α 1) mRNAs (13). However, the RA-associated increase in Era-1 mRNA is only slightly enhanced by concurrent exposure to dibutyryl cAMP (data not shown).

The magnitude of the increase in Era-1 mRNA is dependent on the concentration of RA added exogenously to the F9 stem cells (Fig. 2). The concentration of RA which, 6 hr after addition, induces a half-maximal increase in the Era-1 mRNA level is 2–4 nM. For comparison, the apparent dissociation constant of the F9 cellular RA-binding protein for RA is 9.2 ± 1.1 nM (35). The concentration of RA that induces a half-maximal increase in the laminin B1 mRNA level at 48 hr is also ≈ 4 nM (Fig. 2). Thus, Era-1 mRNA is increased by concentrations of RA that are in the physiologic range, and the fact that the Era-1 dose-response curve correlates with the laminin B1 dose-response curve is consistent with the idea that the early increase in Era-1 mRNA is involved in the subsequent expression of genes such as laminin B1.

The Effect of Protein Synthesis Inhibitors and RA Synthesis Inhibitors on the RA-Associated Increase in the Era-1 mRNA. One of the most striking properties of the Era-1 gene is that the increase in Era-1 mRNA in response to RA is not prevented by protein synthesis inhibitors such as cycloheximide or puromycin under conditions in which greater than 90% of protein synthesis is inhibited (Fig. 3). This demonstrates that protein synthesis is not required for the RA-associated increase in the level of Era-1 mRNA, implying that this increase may be a primary response to RA. In contrast, protein synthesis inhibitors prevent the induction of collagen IV (α 1) and laminin B1 mRNAs by RA (20). The level of Era-1 mRNA is slightly higher after RA plus cycloheximide treatment than after RA treatment alone, and the Era-1 mRNA level in F9 stem cells is also slightly increased by cycloheximide (Fig. 3). This increase may result from the nonspecific protection of mRNAs by ribosomes in the presence of the cycloheximide (36).

Inhibitors of RNA synthesis such as actinomycin D do prevent the RA-associated increase in Era-1 mRNA (Fig. 3, lanes 7 and 8). By examining the steady-state level of Era-1 mRNA after actinomycin D addition, with or without simultaneous addition of RA, an estimate of the relative stability of the Era-1 mRNA in the presence or absence of RA can be obtained. Stability of the Era-1 mRNA is not appreciably altered in the presence of RA (Fig. 3, lanes 9 and 10). When considered together with the cycloheximide results, these actinomycin D results suggest that the RA-associated increase in Era-1 mRNA results, at least in part, from transcriptional activation of the Era-1 gene, rather than from an increase in stability of the Era-1 mRNA associated with the presence of RA.

Within the first 6 hr after RA addition, before the time when the laminin B1 mRNA level begins to increase, the

FIG. 1. Time course of expression of Era-1 mRNA. F9 cells were plated at 1.5×10^6 cells per 150-mm gelatinized tissue culture dish, and after an overnight incubation at 37°C, RA was added (t_0) to a final concentration of 1 μ M (A and C) or 10 nM (B). At various times after RA addition, total cellular RNA was isolated, and 2 μ g was analyzed by RNA blot hybridization as described. 32 P-labeled cDNA insert probes were included at: Era-1, $1-3 \times 10^6$ cpm/ml; laminin B1, 1×10^6 cpm/ml; and actin, 0.5×10^6 cpm/ml. The mouse laminin B1 probe was a 980-bp fragment from pc156 (10). The mouse actin probe was an insert from pAct-1 (33). After washing in $0.2 \times$ SSC at 60°C, blots were exposed to Kodak XAR-5 film at -70°C with an intensifying screen for the following durations: 1–4 days, Era-1; and 2.5–11 hr, laminin B1 and actin. The degree of hybridization was quantitated by scanning densitometry. Values for the Era-1 and laminin B1 mRNAs were normalized to actin mRNA in each lane and plotted as shown below each RNA blot. (A) Era-1, laminin B1, and actin hybridizations and densitometric plot of a RNA blot of F9 RNA isolated at t_0 (lane 1) or at 2 hr (lane 2), 4 hr (lane 3), 6 hr (lane 4), 8 hr (lane 5), 12 hr (lane 6), 24 hr (lane 7), 48 hr (lane 8), and 72 hr (lane 9) after the addition of 1 μ M RA. (B) Hybridization and densitometric plot of a RNA blot of F9 RNA isolated at t_0 (lane 1) or at 4 hr (lane 2), 8 hr (lane 3), 12 hr (lane 4), 24 hr (lane 5), and 48 hr (lane 6) after the addition of 10 nM RA. (C) Hybridization and densitometric plot of a RNA blot of F9 RNA showing the effect of the removal of RA from the growth medium after an 8-hr exposure. RNA was isolated at t_0 (see B, lane 1) or at 4 hr (lane 2), 8 hr (lane 3), 12 hr (lane 4), 24 hr (lane 5) and 48 hr (lane 6) after continuous exposure to 1 μ M RA. Alternatively, RNA was isolated from cells that had been grown for 8 hr in 1 μ M RA, washed three times with medium, and incubated without RA. The lanes contain RNA isolated at 12 hr (relative to t_0) (lane 6), 24 hr (lane 7), or 48 hr (lane 8). In the densitometric plot in C, $\circ$ and $\bullet$ represent points during the continuous exposure to RA, whereas $\triangle$ and $\blacktriangle$ represent points after removal of RA at 8 hr (arrow). A and C are from two separate RNA preparations.

relative stability of the laminin B1 mRNA is also unaffected by the presence of RA (Fig. 3, lanes 7 and 8). In these preliminary experiments, the half-lives of the laminin B1 and Era-1 mRNAs appear shorter than the half-life of actin mRNA, as no significant change in actin steady-state mRNA levels occurs after 6 hr of actinomycin D treatment.

Characterization of the Era-1 Gene. The Era-1 gene encodes a polyadenylated message (Fig. 4A) that is not very abundant even after induction by RA. We estimate that before RA induction, the Era-1 mRNA constitutes $\approx 0.0005\%$ of total F9 stem cell poly(A)⁺ RNA, whereas after RA treatment, Era-1 mRNA reaches $\approx 0.01-0.02\%$ of F9 poly(A)⁺ RNA. This estimate was made by (i) comparison of intensities of bands on RNA gels, exposure times, and the concentrations and lengths of the Era-1 and laminin B1 probes used in the hybridizations, and (ii) the frequency with which Era-1 cDNA clones were found in the RA/cAMP/T 8-hr library. Using more accurate methods, we had previously found the abundance of the laminin B1 mRNA to be

0.76% of the total poly(A)⁺ RNA population in differentiated F9 parietal endoderm cells (13).

The Era-1 gene is a single or low-copy number gene, as Southern analysis of an EcoRI digest of F9 genomic DNA revealed only one band of 8 kb hybridizing to the 5' half of the Era-1 cDNA (Fig. 4B) with an intensity equal to that for c-myc, a single-copy gene (data not shown). When a nearly full-length Era-1 cDNA was used as a probe of an EcoRI F9 genomic digest, two bands of 8 kb and 4 kb were observed (data not shown).

DISCUSSION

The F9 teratocarcinoma stem cell line has been widely used as an *in vitro* model system for the study of cellular differentiation, as well as the mechanism(s) by which RA influences this complex process. We and others (9–15, 17, 18) have previously characterized cDNA clones for cellular genes that show altered expression during the RA-induced

FIG. 2. Dose-response curves for the induction of Era-1 mRNA at 6 hr (A) or laminin B1 mRNA at 48 hr (B) after RA addition. The F9 stem cells were plated and grown as for Fig. 1. At t_0 the following concentrations of RA were added: 0 (lanes 1), 10^{-10} M (lanes 2), 10^{-9} M (lanes 3), 5×10^{-9} M (lanes 4), 10^{-8} M (lanes 5), 5×10^{-8} M (lanes 6), 10^{-7} M (lanes 7), 5×10^{-7} M (lanes 8), 10^{-6} M (lanes 9), and 5×10^{-6} M (lanes 10). At 6 hr (A) and at 48 hr (B) after RA addition, total cellular RNA was isolated and 2 μ g was subjected to RNA analysis. (A) Hybridizations of the Era-1 and actin cDNAs to the RNA at 6 hr. (B) Hybridizations of the laminin B1 and actin cDNAs to the RNA at 48 hr. (C) The hybridizations of the Era-1 cDNA to the 6-hr RA dose-response curve and the laminin B1 cDNA to the 48-hr RA dose-response curve were quantitated by scanning densitometry, normalized to actin mRNA, and plotted.

differentiation of the F9 stem cells, such as those encoding certain homeobox-containing proteins (14), cytoskeletal proteins (15, 17), extracellular matrix proteins (10, 11, 13) and the major histocompatibility antigens (9). The relatively slow

changes in expression of these previously characterized genes following the addition of RA suggests that these changes are indirect effects of RA treatment and that more rapid, direct responses to RA exist.

In this paper, we report the isolation of *Era-1*, a gene that exhibits the properties of a primary target of RA action. The steady-state level of the Era-1 mRNA is rapidly increased after addition of RA to the F9 teratocarcinoma stem cells (Fig. 1A), which is in sharp contrast to previously reported

FIG. 3. Effect of protein synthesis inhibitors and RNA synthesis inhibitors on the induction of Era-1 mRNA by RA. RNA blot analysis of 2 μ g of total cellular RNA from F9 cells grown for 6 hr in medium containing no drugs (lane 1), 1 μ M RA (lane 2), 1 μ g of cycloheximide per ml (lane 3), 1 μ M RA plus cycloheximide at 1 μ g/ml (lane 4), puromycin at 50 μ g/ml (lane 5), 1 μ M RA plus puromycin at 50 μ g/ml (lane 6), actinomycin D at 2 μ g/ml (lane 7), 1 μ M RA plus actinomycin D at 2 μ g/ml (lane 8) or of 5 μ g of total cellular RNA from F9 cells grown for 6 hr in actinomycin D at 2 μ g/ml (lane 9) or 1 μ M RA plus actinomycin D at 2 μ g/ml (lane 10). The blot was hybridized to the Era-1, laminin B1, or actin cDNAs as described. This experiment has been done twice with identical results; exposure times for Era-1 were 2 days (lanes 1–8) and 5 days (lanes 9 and 10). Exposure times for laminin B1 and actin were 6 hr. In the same experiment, either cycloheximide at 1 μ g/ml or puromycin at 50 μ g/ml inhibited the incorporation of [35 S]methionine into trichloroacetic acid-precipitable protein by >90% over the 6-hr time period. Actinomycin D at a concentration of 2 μ g/ml also inhibited the incorporation of [3 H]uridine into trichloroacetic acid-precipitable cellular material by >90% over the 6-hr experiment.

FIG. 4. (A) Total cellular RNA was isolated from F9 cells treated for 12 hr with dibutyryl cyclic AMP (250 μ M) and theophylline (250 μ M) (CT), or with RA (1 μ M), dibutyryl cyclic AMP, and theophylline (RA/cAMP/T; RACT). This RNA was fractionated into poly(A)⁺ and poly(A)⁻ fractions (24). Two micrograms of total unfractionated RNA (T) and 1 μ g of poly(A)⁺ RNA (A⁺) from each condition was fractionated and blotted. The RNA blot was hybridized to the Era-1 cDNA insert. (B) Ten micrograms of F9 genomic DNA was digested with either EcoRI (E), HindIII (H), Pst I (P), or BamHI (B), and analyzed by Southern blotting (32). Blot was hybridized with the 5', 1.25-kb EcoRI fragment of the Era-1 cDNA. Hybridization conditions were the same as those for the RNA blots described in Materials and Methods; exposure time of the autoradiogram was 16 hr.

developmentally regulated genes such as laminin B1, laminin B2, and collagen IV(α 1) (10, 11, 13). In addition, the increase in Era-1 mRNA in the presence of RA does not require concurrent protein synthesis (Fig. 3), indicating that no prior induction of gene expression, at either the transcriptional or translational level, is required for Era-1 mRNA expression. Era-1 mRNA expression is induced by physiologic concentrations of RA in a dose-dependent manner (Fig. 2) and requires the continued presence of RA (Fig. 1C); these are also properties of a primary response to RA.

The question of how retinoids influence gene expression and cell differentiation is unanswered at present, but the discovery of the *Era-1* gene is an important advance in the elucidation of this mechanism. By analogy with the mechanism proposed for steroid hormones, RA complexed to the cellular retinoic acid-binding protein (CRABP) may directly activate the *Era-1* gene, and the *Era-1* gene product may subsequently influence the expression of late genes, such as those encoding laminin and collagen IV in F9 cells. Alternatively, retinoids may regulate gene expression through post-translational modifications of existing proteins that may lead to the increased level of Era-1 mRNA. Other mechanisms of retinoid action may also be postulated, but any other models should take into account this demonstration that retinoids can rapidly modulate mRNA expression in the absence of new protein synthesis. Nuclear run-off transcription assays and further measurements of mRNA stability are in progress to directly determine the relative importance of changes in transcription versus changes in mRNA stability in the RA-associated increase in Era-1 mRNA expression. Analysis of the structures of the *Era-1* gene and RNA should finally permit the elucidation of the initial events in the intracellular action of RA in this F9 model differentiation system.

Although analysis of the *Era-1* gene and RNA should greatly facilitate studies on the mechanism of RA action, several other significant questions with regard to the expression of the Era-1 mRNA require investigation. It is important to determine whether Era-1 mRNA is expressed in other cell types that differentiate in response to RA and to determine whether other genes with the characteristics of a primary response to RA are expressed in F9 teratocarcinoma cells. The questions of when and in what tissues the *Era-1* gene is expressed during mouse embryogenesis can be addressed by *in situ* cDNA-mRNA hybridization experiments with tissue sections of mouse embryos at different developmental stages.

Finally, the further characterization of the putative Era-1 protein product is also of enormous interest. The observations that the Era-1 mRNA does not appear to be very abundant even after induction and that the dose-response of Era-1 mRNA induction at an early time correlates with the dose-response of laminin B1 mRNA induction at a later time after RA addition are consistent with the idea that the *Era-1* gene encodes a protein involved in the control of subsequent gene expression during differentiation. Further analysis of the Era-1 protein product and the introduction of the *Era-1* gene under a heterologous promoter into F9 stem cells will establish whether expression of the Era-1 protein is sufficient for the induction of late genes, such as laminin B1, laminin B2, and collagen IV(α 1) in the absence of RA.

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