Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 6914–6918, August 1993
Biochemistry

E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product

(cell cycle/growth suppressor)

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Communicated by Robert A. Weinberg, April 8, 1993

ABSTRACT **Previous studies have shown that the carboxyl-terminal region of E2F-1 (residues 368–437) can support transcriptional activation when linked to the DNA-binding domain of the yeast transcription factor GAL4. This region also contains an 18-residue retinoblastoma (RB)-binding sequence, raising the possibility that RB binding might inhibit the ability of E2F-1 to form protein–protein contacts required for activation. Here we report a further analysis of the E2F-1 activation domain. In addition, we show that overexpression of RB, but not the RB mutant, RBd22, can inhibit GAL4/E2F-1 activity *in vivo*. Moreover, expression of the simian virus 40 large tumor antigen (T antigen), but not the RB-binding defective T antigen point mutant, K1, can overcome this repression. Three different GAL4/E2F-1 mutants that activate transcription, but fail to bind to RB, are not significantly affected by overexpression of RB. These findings support a model wherein RB suppresses E2F-1-mediated transcriptional activation through direct physical association.**

The product of the retinoblastoma susceptibility gene, pRB, contains a region, or "pocket," capable of forming stable complexes with the transforming proteins encoded by several unrelated DNA tumor viruses (1–4). This same region of pRB is frequently altered in tumors that have sustained loss of function RB gene mutations known to be compatible with stable protein expression. Recent data suggest that the RB pocket can bind to a family of cellular proteins, one of which is the transcription factor E2F (4–9). E2F recognition sequences have been identified in the promoters of a number of genes whose products are thought to play a role in DNA synthesis and/or cell growth control (10, 11). Furthermore, indirect evidence suggests that E2F is "inactive" when bound by RB. For example, transient overexpression of RB *in vivo* leads to a decrease in transcription from reporter plasmids bearing E2F-binding sites (12–15), whereas introduction of E1A into cells leads to an increase in free E2F and an increase in E2F-mediated transcription (16). Thus, E2F is likely to be a downstream target of RB action, and the ability of RB to regulate cell growth may be due, at least in part, to its ability to modulate E2F activity. In keeping with the above, the ability of fragments of the RB polypeptide to suppress the growth of RB−/− osteosarcoma cells has been shown to correlate with their ability to bind to an E2F–DNA complex (17, 18).

A cDNA encoding a cellular RB-binding protein with E2F-like DNA-binding properties has recently been cloned (19–21). This protein, named E2F-1, contains a DNA-binding domain in the amino-terminal region of the protein and a transactivation domain near the carboxyl terminus. The transactivation domain also contains an 18-residue colinear RB-binding sequence (20). Thus, one could envision that RB binding might affect the ability of E2F-1 to form requisite protein–protein interactions necessary for transcriptional activation. Here we report that overexpression of RB can inhibit GAL4/E2F-1 activity *in vivo* and provide genetic evidence that inhibition requires direct association between E2F-1 and RB.

MATERIALS AND METHODS

Plasmid Construction. Expression vectors encoding GAL4 and GAL4/E2F-1 mutants contain the Rous sarcoma virus promoter/enhancer and the simian virus 40 (SV40) poly(A) signal. GAL4, GAL4/E2F-1(368–437), and GAL4/E2F-1(422–437) have been described (19). GAL4/E2F-1(379–437), GAL4/E2F-1(389–437), and GAL4/E2F-1(399–437) were generated by cloning PCR fragments downstream from and in frame with the GAL4 open reading frame in *Bam*HI/*Eco*RI-digested GAL4 plasmid. Carboxyl-terminal deletions were generated by introducing translation stop codons at five amino acid intervals employing site-directed mutagenesis (22). The internal deletions were generated by site-directed loopout mutagenesis. The indicated five codons were deleted and replaced by a *Sca* I restriction site that encodes a Ser and Thr dipeptide. Mutants were screened by restriction analysis and verified by sequencing the entire E2F-1 region.

The reporter plasmid, 3X(GAL4)BG-CAT, was generated by replacing the luciferase gene from pGL2 (Promega) with the β-globin "TATA" box and chloramphenicol acetyltransferase (CAT) gene from pG4CAT (23) and inserting the double-stranded 3X(GAL4) oligonucleotide, 5′-AATTCGGAGGACAGTACTCCGATACGGAGGACAGTACTCCGATACGGAGGACAGTACTCCGT-3′, 5′-CTAGACGGAGTACTGTCCTCCGTATCGGAGTACTGTCCTCCGTATCGGAGTACTGTCCTCCG-3′, upstream from the TATA element.

Cell Culture, Transfections, and CAT Assays. The Burkitt lymphoma cell lines DG75 and Cl-13 were propagated at 37°C in RPMI 1640 medium (GIBCO) containing 10% fetal bovine serum. The osteosarcoma cell line SAOS-2 was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. DG75 and Cl-13 cells were transfected by the DEAE-dextran method as described (23) and SAOS-2 cells were transfected by the calcium phosphate precipitation method (17). The cells were harvested 40 hr after transfection and CAT activity was determined as described (24).

RESULTS

Previous studies employing GAL4/E2F-1 chimeras have mapped the E2F-1 activation domain to a relatively acidic region from residue 368 to 437 (19, 21). A short subdomain within this region (amino acids 409–426) has been shown to be sufficient for binding to RB (20). In an effort to further

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Abbreviations: CAT, chloramphenicol acetyltransferase; SV40, simian virus 40; T antigen, large tumor antigen; RB, retinoblastoma.

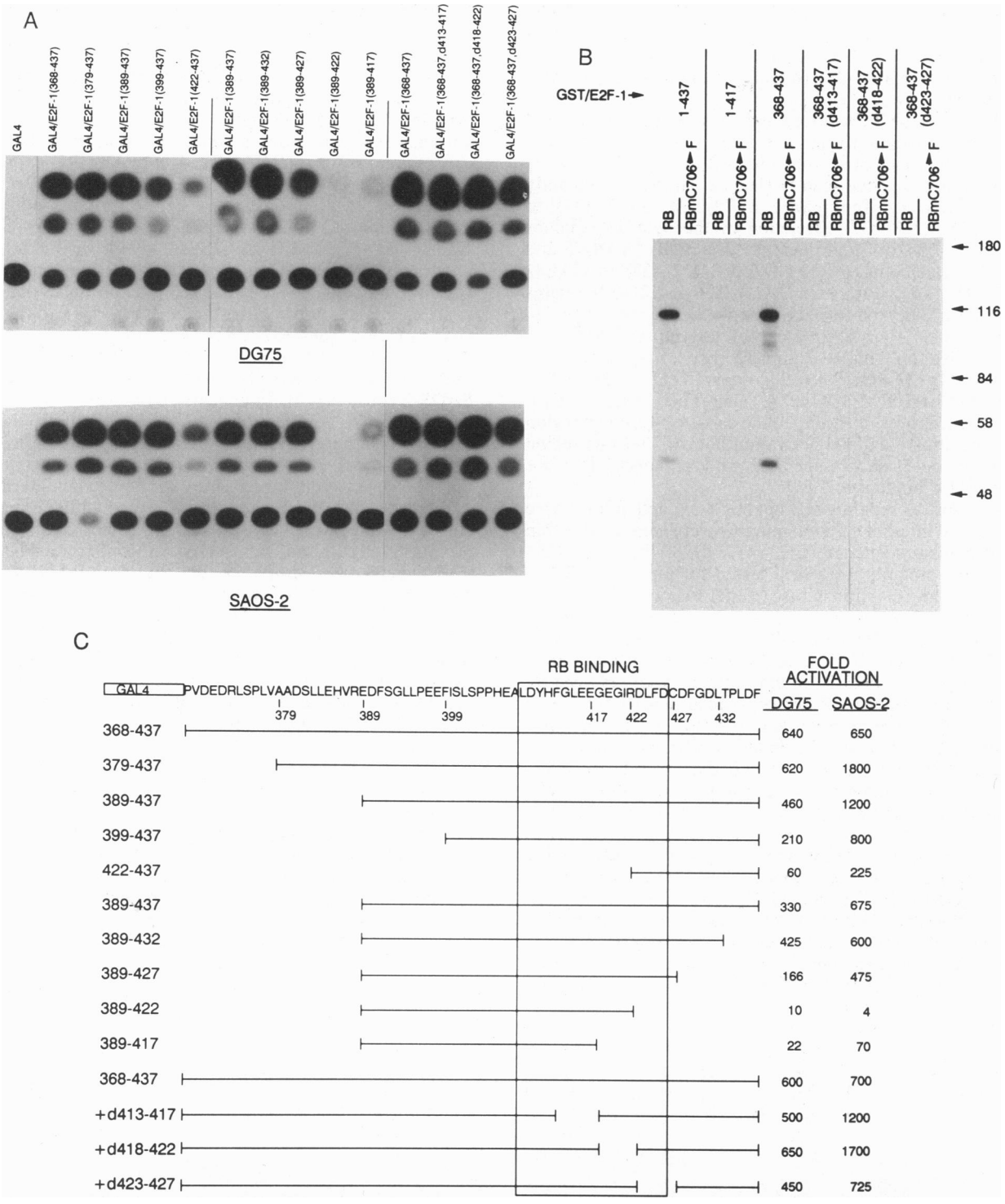

FIG. 1. Deletion analysis of GAL4/E2F-1 proteins. (*A*) The indicated GAL4/E2F-1 expression plasmids were cotransfected with the 3X(GAL4)BG-CAT reporter plasmid. The Burkitt lymphoma cell line DG75 was transfected by DEAE-dextran employing 2 μg of effector plasmid and 2 μg of reporter plasmid. SAOS-2 cells were transfected by the calcium phosphate method using 4 μg of effector plasmid and 4 μg of reporter plasmid. All mutants were tested for DNA binding by electrophoretic mobility shift analysis employing *in vitro* translated proteins and were found to bind with equal affinity (data not shown). (*B*) Analysis of RB binding to E2F-1 mutants. Radiolabeled *in vitro* translated RB or RB mutants (cysteine residue at position 706 is changed to phenylalanine) were incubated with the indicated glutathione *S*-transferase/E2F-1 fusion proteins immobilized on glutathione-Sepharose. The resin was then washed and bound proteins were eluted by boiling in SDS-containing sample buffer, resolved by SDS/polyacrylamide gel electrophoresis, and detected by fluorography. (*C*) Schematic representation and activities of GAL4/E2F-1 deletion mutants. Quantitation was performed using a Betagen Betascope and fold activation represents the activities relative to GAL4 activity. Activities for some of the effectors were beyond the linear range of the assay and should be considered approximate values.

define the E2F-1 activation domain and its relationship to the RB-binding domain, a more extensive GAL4/E2F-1 deletion series was analyzed for transcriptional activation. For these studies, the Burkitt lymphoma cell line DG75, which was used for the previous mapping study, and the RB−/− osteosarcoma cell line SAOS-2 were employed. A series of effector plasmids, shown schematically in Fig. 1*C*, were cotransfected with a reporter plasmid containing three

GAL4-binding sites upstream from the β-globin TATA element [3X(GAL4)BG-CAT]. The amino- and carboxyl-terminal GAL4/E2F-1 deletion mutants were expressed at comparable levels as determined by immunoblot analysis (data not shown).

Although the acidic residues in the region from residue 368 to 437 are distributed relatively uniformly, an asymmetric activity profile was observed (Fig. 1 *A* and *C*). The first 31 amino-terminal residues can be deleted without significantly affecting activity [compare GAL4/E2F-1(399–437) to GAL4/E2F-1(368–437)]. Deletion of the next 23 residues resulted in a 3- to 4-fold decrease in activity in DG75 and SAOS-2 cells [compare GAL4/E2F-1(422–437) to GAL4/E2F-1(399–437)]; however, GAL4/E2F-1(422–437) retains significant activity (60-fold activation in DG75 and 225-fold in SAOS-2 cells). Analysis of a carboxyl-terminal deletion series revealed an almost complete loss of activity when residues 423–427 were deleted [compare GAL4/E2F-1(389–427) to GAL4/E2F-1(389–422)]. Therefore, crucial activation sequences appear to map to the carboxyl-terminal region of the previously defined activation domain and this region appears to be closely associated with sequences that are important for binding to RB.

In an effort to separate RB binding from activation, three 5-amino acid internal deletion mutants were generated within the RB-binding sequence (d413–417, d418–422, and d423–427). These mutants were first tested for binding to RB *in vitro*. Glutathione *S*-transferase/E2F-1 fusion proteins were expressed in *Escherichia coli*, recovered on glutathione-Sepharose, and incubated with *in vitro* translated RB or RBm706F [a loss of function RB mutant identified in a small cell lung carcinoma (25)]. Bound proteins were resolved by SDS/polyacrylamide gel electrophoresis and detected by fluorography. As expected, RB, but not the RB mutant, RBm706F, bound to the full-length E2F-1 chimera and to GST-E2F-1(368–437) (Fig. 1*B*). RB did not bind to GST-E2F-1(368–437) chimeras bearing any of the above three internal deletion mutations nor did it bind to GST/E2F-1(1-417).

As shown in Fig. 1*A*, the activity of all three RB-binding mutants was comparable to that of the parental nonmutated protein [GAL4/E2F-1(368–437)]. The observation that GAL4/E2F-1(368–437)(d423–427) is active was unexpected since the carboxyl-terminal deletion series described above mapped this region as being important for activation. However, subsequent deletion studies suggest that a functionally redundant element is localized carboxyl terminal to residue 427 and both regions must be deleted in order to abrogate activity (data not shown).

To directly assess whether RB can inhibit E2F-1 transcriptional activation, plasmids expressing GAL4/E2F-1 fusion proteins with intact or mutated RB-binding domains were cotransfected with either a control plasmid (pCMV), an RB expression vector (pCMV-RB), or a mutant RB expression vector (pCMV-RBd22) and the 3X(GAL4)BG-CAT reporter plasmid. Activation by GAL4/E2F-1 proteins containing intact RB-binding domains [GAL4/E2F-1(285–437), GAL4/E2F-1(368–437), and GAL4/E2F-1(389–437)] was inhibited by overexpression of RB, but not the RB mutant, in the Burkitt lymphoma cell line Cl-13 (Fig. 2). Mutants that failed to bind RB *in vitro* [GAL4/E2F-1(368–437) (d413–417), GAL4/E2F-1(368–437)(d418–422), GAL4/E2F-1(368–437)(d423–427)], however, were not significantly affected by overexpression of RB. Similar results were observed in SAOS-2 cells (Fig. 3). In contrast, overexpression of RB in DG75 cells had no effect on GAL4/E2F-1 activity (data not shown). Whether this failure of RB to inhibit GAL4/E2F-1 in DG75 cells was due to technical reasons or due to biologic differences between the cell lines remains to be determined.

It has recently been shown that short synthetic peptides corresponding to the RB-binding sequences found in viral proteins such as T and E1A can block the interaction between E2F-1 and RB *in vitro* (19). We therefore asked whether overexpression of T antigen could block the ability of RB to suppress E2F-1-mediated transactivation. As a control, the stable T antigen mutant K1, which fails to bind RB by virtue of a single amino acid substitution (26), was studied in parallel. As can be seen in Fig. 3, T antigen, but not K1, was capable of blocking the ability of RB to suppress GAL4/E2F-1(368–437) transactivation.

DISCUSSION

The data presented here strongly suggest that RB can suppress the ability of E2F-1 to serve as a transactivator and are consistent with the notion that E2F-1 is a downstream target of RB action. The observation that three different E2F-1 mutants that failed to bind RB were not affected by RB overexpression suggests that suppression is mediated by direct interactions between E2F-1 and RB and not through indirect cell-cycle effects. This effect does not require an alteration of E2F-1 DNA-binding activity, although this, as

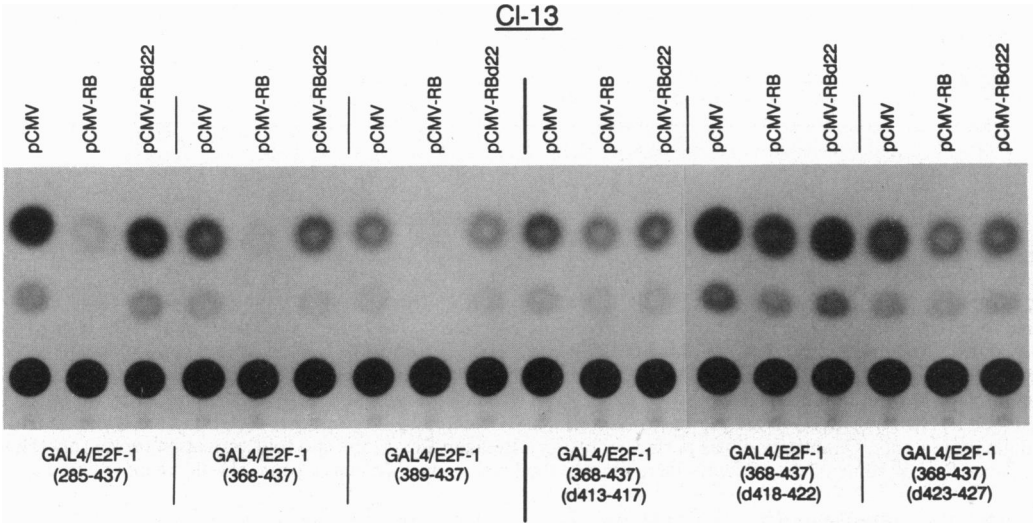

FIG. 2. Overexpression of RB specifically inhibits GAL4/E2F-1 activity *in vivo*. Cl-13 cells were transfected by the DEAE-dextran method employing 2 μg of 3X(GAL4)BG-CAT, 2 μg of the indicated GAL4/E2F-1 effector plasmid, and 10 μg of either a control plasmid, pCMV, the RB expression plasmid pCMV-RB, or the RB mutant expression plasmid pCMV-RBd22 (17).

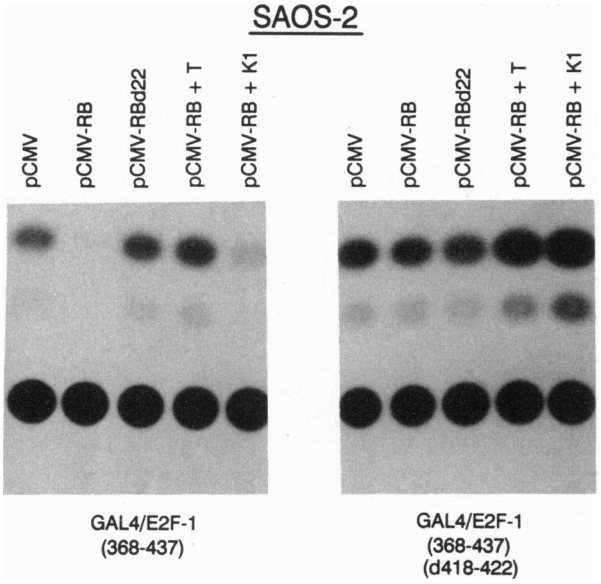

FIG. 3. RB suppression of GAL4/E2F-1(368–437) activity in SAOS-2 cells and the release from RB suppression by SV40 large tumor antigen (T antigen). Transfections were performed using 4 μg of reporter plasmid [3X(GAL4)BG-CAT], 4 μg of either GAL4/E2F-1(368–437) or GAL4/E2F-1(368–437) (d418–422), and 12 μg of the indicated RB or RB control plasmids. In addition, 12 μg of SV40 early promoter/enhancer-driven T or K1 expression vectors (generous gifts from James A. DeCaprio) were added where indicated. Twelve micrograms of a control plasmid containing only the SV40 promoter and enhancer was added to transfections lacking the T or K1 expression plasmids.

proposed by others (6), may also play a role in the regulation of E2F-1 by RB. The finding that T antigen, but not K1, could overcome this effect is consistent with earlier studies that suggested that viral oncoproteins such as E1A, in a conserved region 2-dependent manner, led to dissociation of RB–E2F complexes with subsequent liberation of a transcriptionally active form of E2F (16, 27). Our data are also in keeping with earlier reports that showed that cotransfection of wild-type, but not mutant, RB with reporters bearing E2F DNA-binding sites led to a decrease in reporter activity (12, 13). These studies, though consistent with the view that RB suppresses E2F-mediated transactivation, were potentially confounded by RB- and E1A-mediated cell cycle effects. For example, one could imagine that the results obtained upon introduction of RB were an indirect consequence of RB-mediated growth suppression. In particular, one could speculate that RB led to an arrest of cells in a phase (or phases) of the cell cycle when E2F does not appear to be active (for example, G_1). Such a hypothesis would be consistent with existing data regarding the effect of introducing wild-type RB into RB−/− cells (17, 28–31). Furthermore, these results must now be interpreted in light of data that suggest that there is more than one E2F species (ref. 33; J. Lees, K. Helin, and E. Harlow, personal communication; S. Field, M. Greenberg, and W.G.K., unpublished data)—that is to say, the readout from an E2F-driven reporter may reflect transactivation from a family of polypeptides, only one (or perhaps a subset) of which is a direct target of RB action. By linking the E2F-1 activation domain to a heterologous DNA-binding domain from the yeast transcription factor GAL4, we have attempted to circumvent complications imposed by other E2F family members. Nonetheless, we recognize that the GAL4/E2F-1 fusion proteins studied here may not interact with RB in precisely the same manner as endogenous E2F proteins.

The spatial relationship between E2F-1 activation sequences and the RB-binding domain strongly suggest that binding of RB to E2F-1 prevents interactions with factors that are required for transcriptional activation. That binding to RB, or perhaps a similar pocket protein [such as p107 (32)], is not required for E2F-1 transactivation was shown by mutants such as GAL4/E2F-1(368–437) (d413–417), GAL4/E2F-1(368–437) (d418–422), and GAL4/E2F-1(368–437) (d423–427), which no longer bind to RB and p107 yet can serve as potent transactivators. This result is consistent with the observation of others (21). Indeed, the finding that RB binding and transactivation are dissociable functions raises the possibility that some human tumors will be found to contain E2F-1 mutants that have escaped RB control as a result of subtle mutations in their RB-binding sequences.

We thank Jason Harris for excellent technical assistance, Fang Liu and Michael Green for their generous gift of anti-GAL4 antibody, and Drs. D. Livingston and C. Cayrol for critical comments on the manuscript. We also give special thanks to David Livingston for his continued support. This work was supported by Physician Scientist Award K11 CA01528-03 (W.G.K.), U.S. Public Health Service Grant 5RO1 CA-43143 from the National Institutes of Health (S.H.S.), and a Leukemia Society Special Fellowship awarded to E.K.F.

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