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Transcriptional Stimulation of the Retina-Specific *QR1* Gene upon Growth Arrest Involves a Maf-Related Protein

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The avian neural retina (NR) is derived from proliferating neuroectodermal precursors which differentiate after terminal mitosis and become organized in cell strata. Proliferation of postmitotic NR cells can be induced by infection with Rous sarcoma virus (RSV) and requires the expression of a functional v-Src protein. *QR1* is a retina-specific gene expressed exclusively at the stage of growth arrest and differentiation during retinal development. In NR cells infected with *ts*PA101, an RSV mutant conditionally defective in pp60$^{v\text{-}src}$ mitogenic capacity, *QR1* expression is downregulated in proliferating cells at 37°C and is fully restored when the cells become quiescent as a result of pp60$^{v\text{-}src}$ inactivation at 41°C. We were able to arrest proliferation of *ts*PA101-infected quail NR cells expressing an active v-Src protein by serum starvation at 37°C. This allowed us to investigate the role of cell growth in regulating *QR1* transcription. We report that *QR1* transcription is stimulated in growth-arrested cells at 37°C compared with that in proliferating cells maintained at the same temperature. Growth arrest-dependent stimulation of *QR1* transcription requires the integrity of the A box, a previously characterized *cis*-acting element responsible for *QR1* transcriptional stimulation upon v-Src inactivation and during retinal differentiation. We also show that formation of the C1 complex on the A box is increased upon growth arrest by serum starvation in the presence of an active v-Src oncoprotein. Thus, the C1 complex represents an important link between cell cycle and developmental control of *QR1* gene transcription during NR differentiation and RSV infection. By using antibodies directed against different Maf proteins of the leucine zipper family and competition with Maf consensus site-containing oligonucleotides in a gel shift assay, we show that the C1 complex is likely to contain a Maf-related protein. We also show that a purified bacterially expressed v-Maf protein is able to bind the A box and that the level of a 43-kDa Maf-related protein is increased upon growth arrest in infected retinal cells. Moreover, ectopic expression of c-*maf*I, c-*maf*II, and *maf*B cDNAs in quiescent *ts*PA101-infected quail NR cells is able to stimulate transcription of a *QR1* reporter gene through the A box. Therefore, *QR1* appears to be the first target gene for a Maf-related protein(s) in the NR.

A major challenge in studying vertebrate development is to understand the tight balance between proliferation-quiescence checkpoints and differentiation of terminally postmitotic cells. The relationship between growth arrest and establishment of differentiation programs and their timing with respect to one another is poorly characterized. The molecular basis for the transition of cells from "in cycle" to "out of cycle" (G_0) also is not well understood, although various molecules involved in the regulation of cell cycle checkpoints have recently been identified. Failure to enter quiescence can result in blocking of differentiation and developmental programs as well as in malignant transformation.

The avian neural retina (NR) provides a useful system for studying the balance between growth control, differentiation, and spatial organization during development of a mature organ. The NR is a laminated structure composed of different cell types which derive from proliferating neuroectodermal precursors. Each cell type withdraws from the cell cycle in a defined temporal distribution, reaches its final destination, and completes differentiation after terminal mitosis (6, 42).

As a result of pp60$^{v\text{-}src}$ expression, postmitotic NR cells are induced to proliferate in vitro (60). Whereas expression of the c-*src* gene is regulated during NR development and coincides with differentiation (74, 80), the v-*src* gene product has been shown to interfere with the differentiation programs of various specialized cell types, including NR cells (2, 25, 33, 43, 53, 73, 78, 79). This effect could be mediated by forcing the cells to reenter the cell cycle, through activation of immediate-early genes (28, 41), as well as by shutting off expression of differentiation-specific genes (24, 32, 33, 36, 79) or growth arrest-specific genes (7, 71).

Several genes have been shown to be specifically expressed upon growth arrest in fibroblasts (7, 10, 16, 17, 21, 27, 34, 56, 71). They are likely to play an important role in controlling the transition from proliferation to quiescence (21). Since they are widely expressed, an interesting question is whether they are involved in more-restricted differentiation programs or whether they are replaced by other cell-type-specific or tissue-specific "growth arrest" genes in specialized tissues. Most of the growth arrest-specific genes appear to be regulated at the posttranscriptional level (15, 34). Quiescence-dependent transcriptional stimulation of the p20k (57) and *gas*1 (22) promoters has been more extensively investigated. However, little is known about the *cis*-acting elements and cognate transcription factors that mediate growth arrest-specific transcription.

We previously reported the isolation of an NR-specific gene, named *QR1*, the expression of which coincides with terminal mitosis and differentiation during avian embryogenesis. Expression of *QR1* begins when the last NR cell types to divide

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undergo the final set of divisions and program terminal differentiation. Its expression is detected exclusively in Müller (glial) cells. *QR1* transcription is maintained throughout differentiation, and it is abruptly shut off when the complete development of the retina is achieved (32). The *QR1* gene product is a secreted glycoprotein which displays sequence similarity in its C-terminal moiety with the osteonectin/SPARC protein (54, 69) and is associated with the extracellular matrix. Studies on its localization in the developing retina suggest that it may play a role in photoreceptor differentiation (13). Taken together, our data indicate that *QR1* is a marker of NR differentiation.

Transcription of the *QR1* gene is downregulated when postmitotic quail NR (QNR) cells are induced to proliferate by temperature-sensitive mutants of the v-Src oncoprotein at the permissive temperature (37°C) and is fully restored when these cells cease to divide upon inactivation of pp60$^{v\text{-}src}$ at the nonpermissive temperature (41°C) (32). In addition, studies with conditional v-*src* mutants that induce NR cell division in the absence of morphological transformation (12) suggested that the negative control of *QR1* gene expression is primarily correlated with the mitogenic activity of the v-Src oncoprotein (32). Therefore, *QR1* transcription appears to be correlated with cell quiescence both in ovo and in cells conditionally induced to multiply by v-*src* mutants.

We previously showed that transcriptional downregulation of *QR1* expression by the v-*src* gene product involves a *cis*-acting regulatory region named the A box (63). This regulatory region also mediates stimulation of *QR1* transcription during retinal development and differentiation (64). We also identified a developmentally regulated DNA-protein complex (C1) involved in such regulation. Formation of the C1 complex specifically increases during terminal differentiation of postmitotic retinal cells and decreases upon induction of NR cell proliferation by the v-Src protein (63, 64).

Little is known about the factors involved in transcriptional repression by pp60$^{v\text{-}src}$. In contrast, several studies have investigated the mechanisms that lead to transcriptional stimulation of genes by the v-Src protein (5, 9, 20, 23, 35, 37, 67, 70, 82). In several cases, positive regulation of transcription involves TRE or CRE elements and their cognate transcription factors of the leucine zipper family. The prototypes of this family are the Jun/Fos proteins (1). Several members of a new gene family encoding leucine zipper proteins, the *maf* genes, have been recently characterized (59). The Maf proteins are divided in two subfamilies: (i) that of members containing an acidic domain in the amino-terminal portion, the transactivating domain (48), such as c-Maf, MafB, Kr, and the retina-specific protein Nrl (18, 26, 44, 46, 52, 75), and (ii) that of the small members lacking this domain, such as MafF, MafG, MafK, and p18 (4, 29, 40, 45). The in vivo functions of most of these proteins are still unknown. Interestingly, the small members of this family have been implicated in tissue-specific transcription through dimerization with a tissue-specific transcription factor(s) (4, 40). Moreover, the *maf*-related gene *kr* was shown to be involved in development of the nervous system (18), and the *maf* proto-oncogene was shown to be involved in transcriptional stimulation of the Purkinje cell-specific *L7* gene (52).

In this work, we studied the mechanisms by which v-Src interferes with NR differentiation. Specifically, we investigated the possibility that downregulation of *QR1* transcription by pp60$^{v\text{-}src}$ may be a direct consequence of its mitogenic capacity and that cell quiescence per se may be sufficient to activate expression of this gene.

We report that cell quiescence is indeed able to stimulate transcription of *QR1* and that this effect involves the A box. This suggests that *QR1* is a growth arrest NR-specific gene and that the A box represents a "quiescence-responsive unit" (QRU) in the transcriptional regulatory region of this gene. We show that formation of the C1 complex on the A box is induced upon NR cell growth arrest and that a protein(s) related to the *maf* gene family appears to be involved in both formation of this complex and activation of *QR1* transcription. Taken together, these results suggest that the C1 complex is a potential candidate for integrating signals that result in withdrawal from the cell cycle and establishment of differentiation in the developing retina.

MATERIALS AND METHODS

Cells and viruses. QNR cells were dissected from 8-day-old quail (*Coturnix coturnix japonica*) embryos as described previously (61). QNR cells were cultured in Eagle basal medium supplemented with 10 or 0.1% fetal calf serum (FCS). The isolation and characterization of the temperature-sensitive mutants *ts*NY68 (49) and *ts*PA101 (12) have been previously described. QNR cells were infected as previously reported (60). For examination of the growth kinetics, cells were plated at a density of 2×10^5 per 60-mm-diameter petri dish in medium containing 10% FCS at 37°C. One day later, the medium was replaced, and the cells were maintained in 0.1 or 10% FCS at 37°C or in 10% FCS at 41°C. The cell numbers in two plates per time point were counted at regular intervals in a Thomas densitometer.

Northern (RNA) blot analysis. Total RNA was isolated by using the guanidium thiocyanate-cesium chloride method (14). Twenty micrograms of total RNA was denatured at 50°C in a formamide-formaldehyde mixture, separated by electrophoresis on a 1% agarose–2.2 M formaldehyde gel (55), and transferred to nitrocellulose (77). The membranes were incubated with a 900-bp *Eco*RI fragment of the *QR1* cDNA labeled by nick translation. The filters were also hybridized with the glyceraldehyde-3-phosphate dehydrogenase avian probe to assess the amount of RNA.

Plasmids. The CAT5/*QR1* reporter constructs used for the experiments shown in Fig. 3 and 10 were previously described (63). The four-times-multimerized wild-type (4×WT) and mutated-A-box (4×A$^-$) plasmids were constructed as follows. The −1208 WT int oligonucleotide (5′-CCTCTCCCTTCAGACGCT GACTAGCTGACAGGACAGCAGAATTTCAAC-3′) or the same oligonucleotide mutated in the two repeats of the A box (5′-CCTCTCCCTTCAGACGC**G** **T**ACTAGC**GT**ACAGGACAGCAGAATTTCAAC-3′) (−1208 A$^-$ int) was phosphorylated, multimerized by ligation, and subcloned upstream of the −109 thymidine kinase (TK) promoter (TK10) (76) in the forward orientation. The eukaryotic expression plasmids pEF BssHII, *maf*B/pEF BssHII (44), and *maf*K/pEF BssHII (40) were previously described. The c-*maf*I gene was previously described (46). The c-MafII protein is a newly characterized isoform encoded by the c-*maf* gene which lacks the last 10 carboxy-terminal amino acid residues. The eukaryotic expression plasmid c-*maf*I/pEF BssHII was constructed by subcloning an *Mlu*I fragment of the previously described c-*maf* gene clone (46) into a unique *Bss*HII site of the pEF BssHII plasmid, a derivative of the pEF Bos vector (58). The c-*maf*II/pEF BssHII plasmid was constructed by replacing the 3′ region of c-*maf*I with the corresponding part of the type II c-*maf* cDNA sequence.

Transient cell transfections and CAT assays. One day prior to transfection, *ts*PA101-infected QNR cells were seeded at 6×10^5 cells per 100-mm-diameter petri dish in medium supplemented with 10% FCS and maintained at 37°C. Cells were transfected with 10 μg of CAT5/*QR1* reporter plasmid, 2 μg of β-actin–*lacZ* (8), and 8 μg of carrier DNA essentially as previously described (63) with the following modifications. Five hours after transfection, the precipitates were washed three times with phosphate-buffered saline. For each reporter construct, three plates were transfected. After washing, one plate was incubated with medium containing 0.1% FCS at 37°C, and the other two plates were incubated with medium containing 10% FCS at 37 and 41°C, respectively. The cells were maintained under these conditions for 96 h. Preparation of cell extracts and chloramphenicol acetyltransferase (CAT) assays were then performed as previously described (63). Extracts were normalized for β-galactosidase activity (3) and protein concentration. CAT assays were performed on extracts containing equal amounts of β-galactosidase activity. Quantification of CAT activities was performed by scintillation counting. For the experiments shown in Fig. 10 and Table 1, 3 μg of the different *maf* gene expression vectors or of the pEF vector was cotransfected together with 10 μg of CAT5/*QR1* or 1 μg of TK/*QR1* reporter construct. Cells were maintained at 41°C in 10% FCS for 48 h. An average of three to five independent experiments were routinely examined.

Nuclear extract preparation and gel retardation assays. Nuclear extracts were prepared from *ts*PA101-infected cells maintained at 41°C in 10% FCS or at 37°C in 10 or 0.1% FCS, essentially as previously described (63) with the following modifications. The different buffers were supplemented with 0.75 mM spermidine and 0.15 mM spermine. After cytoplasmic lysis, sucrose was added to a final concentration of 0.4 M. Lysates were subsequently layered on a 0.5 M sucrose cushion and centrifuged at 4°C for 10 min at 1,500 × *g*.

For DNA-protein binding reaction mixtures (20 μl), 0.5 ng of ^{32}P-labeled kinase-treated double-stranded −1208 WT int oligonucleotide (see above for the

sequence), spanning from position −1208 to −1161, was mixed on ice with 0.4 μg of poly(dI-dC) (Pharmacia), 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 60 mM KCl, 4% Ficoll, and 1 to 2 μg of nuclear extract. Two different batches of poly(dI-dC) were used for the experiments in Fig. 4, 5, and 6, which is responsible for the different ratios between formation of the C1 and C2 complexes observed in these figures. For the experiments shown in Fig. 4B and 5B, the following oligonucleotides were used: 5′-CCTCTCCCT TCAGACGCTGACTAGCTGAC-3′ (WT) as the *QR1* WT competitor, 5′-CCT CTCCCTTCAGACGC**GT**ACTAGC**GT**AC-3′ (A⁻) as an oligonucleotide mutant in the two repeats (63), and 5′-CACTTTCCTCTTTCCGTAAGCGTCT GTTTACCCTGGGAT-3′ (p20k) as an oligonucleotide containing the QRU of the p20k gene (57). Competition experiments included a molar excess (2, 10, or 50 times) of unlabeled, double-stranded oligonucleotide in the reaction mixture. For the experiments shown in Fig. 9, oligonucleotides 1, 2, 23, and 29 (47) and the −1005 M (nonspecific [NS]) oligonucleotide (63) were used as unlabeled DNA competitors in 8-, 40-, and 200-times molar excesses. For the experiments shown in Fig. 8, labeled oligonucleotides 1, 23, and *QR1* WT and A⁻ were used as probes and incubated with a bacterially expressed maltose-binding protein–v-Maf fusion protein, which was purified as described previously (47). For the experiments shown in Fig. 6 and 8, nuclear extracts were preincubated for 15 min at 4°C with the different sera, and then the labeled −1208 WT int or 1 probe was added and left for an additional 15 min at room temperature. The anti-v-Maf (46) and anti-MafK (29) polyclonal sera were previously described. The MafB polyclonal antiserum was prepared as follows. Since the intact MafB protein was highly toxic to bacterial cells, a deletion mutant, MafB MD26.20, was expressed by using the *Escherichia coli* expression vector pET-3b (68). The MafB MD26.20 mutant protein contains a deletion of seven amino acid residues in its DNA binding domain (Lys-229–Gln–Lys–Arg–Arg–Thr–Leu-235) and was prepared by replacing a restriction fragment (*Sau*3AI-2721–*Mlu*I-2791) of the chicken *maf*B gene (pRAM-B-GEM) (44) with a double-stranded synthetic oligonucleotide. An *Nco*I-*Bss*HII fragment from this plasmid, deleting the N-terminal 17 amino acid residues of the MafB protein, was subcloned into the *Bam*HI site of the pET-3b plasmid. Thus, the first 17 amino acids of the MafB protein were replaced by 15 amino acids derived from the expression vector and *Bam*HI linker sequences used for subcloning. Induction of expression and purification of the recombinant protein were performed as previously described (46). Because of the high degree of similarity of the v-Maf and MafB proteins, the anti-v-Maf and anti-MafB sera cross-react in their specificity (48). The MafK protein was reported not to recognize the highly similar MafF protein synthesized in vitro (29).

Immunoblotting. Nuclear extracts prepared as described above were resolved by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE) and transferred to nitrocellulose membrane (Schleicher & Schuell). The membranes were incubated with anti-v-Maf serum (46), anti-v-Maf serum preincubated for 2 h at room temperature with the antigen used to inject the rabbits, or anti-MafB serum diluted 1:2,000. Proteins were detected by chemiluminescence under the conditions recommended by the supplier (Amersham).

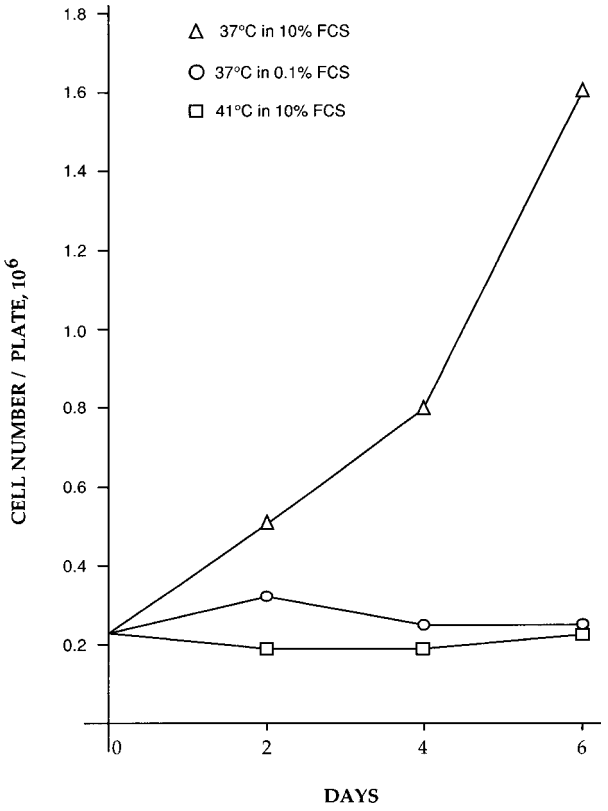


FIG. 1. Division of NR cells infected with the *ts*PA101 virus can be arrested at 37°C by serum starvation. Cells were plated at a density of 2×10^5 cells per 60-mm-diameter petri dish in medium containing 10% FCS. Twenty-four hours later (day 0), the cells were maintained under the indicated conditions. Cell numbers in duplicate dishes were counted at the indicated times.

RESULTS

***QR1* gene expression is controlled by growth arrest.** Cultures of QNR cells from 8-day-old embryos are composed of neuronal and glial cells that rapidly cease to divide and cannot be propagated under our culture conditions (Eagle basal medium supplemented with 10% FCS) (19). Upon infection with the temperature-sensitive mutant *ts*PA101 (12), QNR cells acquire a sustained proliferative capacity at 37°C in the presence of an active v-*src* gene product but are not morphologically transformed. At the nonpermissive temperature (41°C), v-Src is inactivated and cells stop dividing (11, 65). *QR1* gene transcription is strongly downregulated in proliferating cells at 37°C and resumes at 41°C to reach levels comparable to those found in postmitotic QNR cells in ovo (32).

To study the relationship between cell quiescence and *QR1* expression, we first investigated whether division of *ts*PA101-infected cells could be arrested at the permissive temperature by maintaining them in medium containing a low serum concentration. Therefore, *ts*PA101-infected QNR cells were seeded at a low density in medium containing 10% FCS. One day later, cultures were placed under various conditions of serum concentration and temperature, and cell numbers were counted at regular intervals (Fig. 1). Only cells maintained in 10% FCS at 37°C were able to sustain division, whereas cells maintained in 0.1% FCS at 37°C stopped dividing after 4 days of serum starvation. During the first 2 days, corresponding to the initial exponential phase, the number of cells maintained in 10% FCS at 37°C doubled in about 40 h. In contrast, the cultures maintained in 0.1% FCS at 37°C displayed a markedly reduced growth rate. Thus, it is possible to arrest division of *ts*PA101-infected NR cells by serum starvation, despite the presence of an active v-Src protein. This result is in agreement with those of previous experiments showing that division of QNR cells infected with the *ts*NY68 virus can be arrested by serum starvation (30, 66). The *ts*NY68 virus has mitogenic properties similar to those of the *ts*PA101 virus except that it induces morphological transformation at the permissive temperature.

To investigate the effect of cellular quiescence on *QR1* expression, we analyzed by Northern blotting RNAs extracted from *ts*PA101-infected QNR cells maintained at 41°C in 10% FCS or at 37°C in 10 or 0.1% FCS for 2 and 4 days (Fig. 2). As previously reported (32), the *QR1* mRNA was weakly detectable in proliferating QNR cells at 37°C (lane 1). We did not detect an increase of the *QR1* transcript after 2 days of serum starvation at 37°C (Fig. 2; compare lanes 1 and 2), before cell growth was completely arrested, but we reproducibly detected an increase in the level of *QR1* transcript after 4 days (lane 3). However, the mRNA level was significantly lower than that found in QNR cells rendered quiescent by $pp60^{v\text{-}src}$ inactivation at 41°C (compare lanes 3 and 4). Similarly, a partial induction of *QR1* expression was observed in *ts*PA101-infected confluent QNR cultures maintained at 37°C in 10% FCS and growth arrested by contact inhibition, as well as in *ts*NY68-infected cells growth arrested by serum depletion at the per-

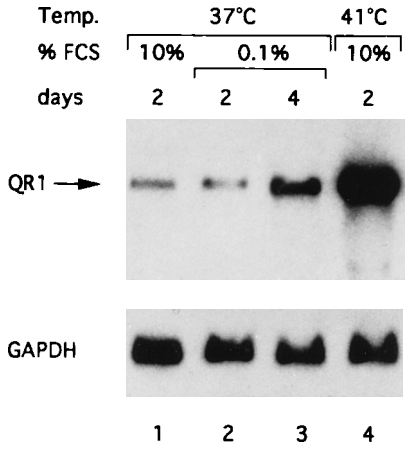


FIG. 2. Induction of *QR1* mRNA in quiescent *ts*PA101-infected QNR cells expressing an active v-Src oncoprotein. Twenty micrograms of total RNA extracted from *ts*PA101-infected QNR cells maintained for 2 days in 10% FCS at 37°C (lane 1), in 0.1% FCS at 37°C (lane 2), and in 10% FCS at 41°C (lane 4) and for 4 days in 0.1% FCS at 37°C (lane 3) was analyzed by Northern blotting and hybridized with a radioactively labeled *QR1* cDNA probe. The same membrane was also hybridized with an avian glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe to assess the amount of RNA. Temp., temperature.

missive temperature (data not shown). Accordingly, we also detected a partial increase in the *QR1* protein in infected QNR cells growth arrested by serum starvation at 37°C (data not shown). Therefore, the *QR1* gene can be considered a quiescence-responsive gene. However, in NR cells expressing the v-Src protein, both growth arrest and $pp60^{v\text{-}src}$ inactivation are required to restore *QR1* expression at its maximal level.

Growth arrest regulates *QR1* expression at the transcriptional level. To determine whether the *QR1* 5′ flanking sequences were able to confer cell quiescence-dependent expression to the CAT reporter gene, constructs containing various lengths of these upstream sequences (63) were transfected into *ts*PA101-infected QNR cells maintained at 37°C in 10% FCS. Five hours after transfection, cells were transferred to fresh medium containing 10 or 0.1% FCS at 37°C or to medium containing 10% FCS and incubated at 41°C. Cell extracts were tested for their CAT activity 96 h after transfection.

A construct containing 1,265 bp of *QR1* 5′ flanking sequences (position −1265 to +55 [−1265/+55]) showed higher-level transcriptional activity in cells growth arrested by serum starvation (Fig. 3) than in proliferating cells maintained at 37°C in 10% FCS. However, the transcriptional activity of the −1265/+55 construct in serum-starved cells at 37°C did not reach the levels obtained in quiescent cells upon inactivation of the v-Src oncoprotein at 41°C. Thus, a *cis*-acting element(s) capable of responding to growth arrest is present in the upstream regulatory regions of the *QR1* gene.

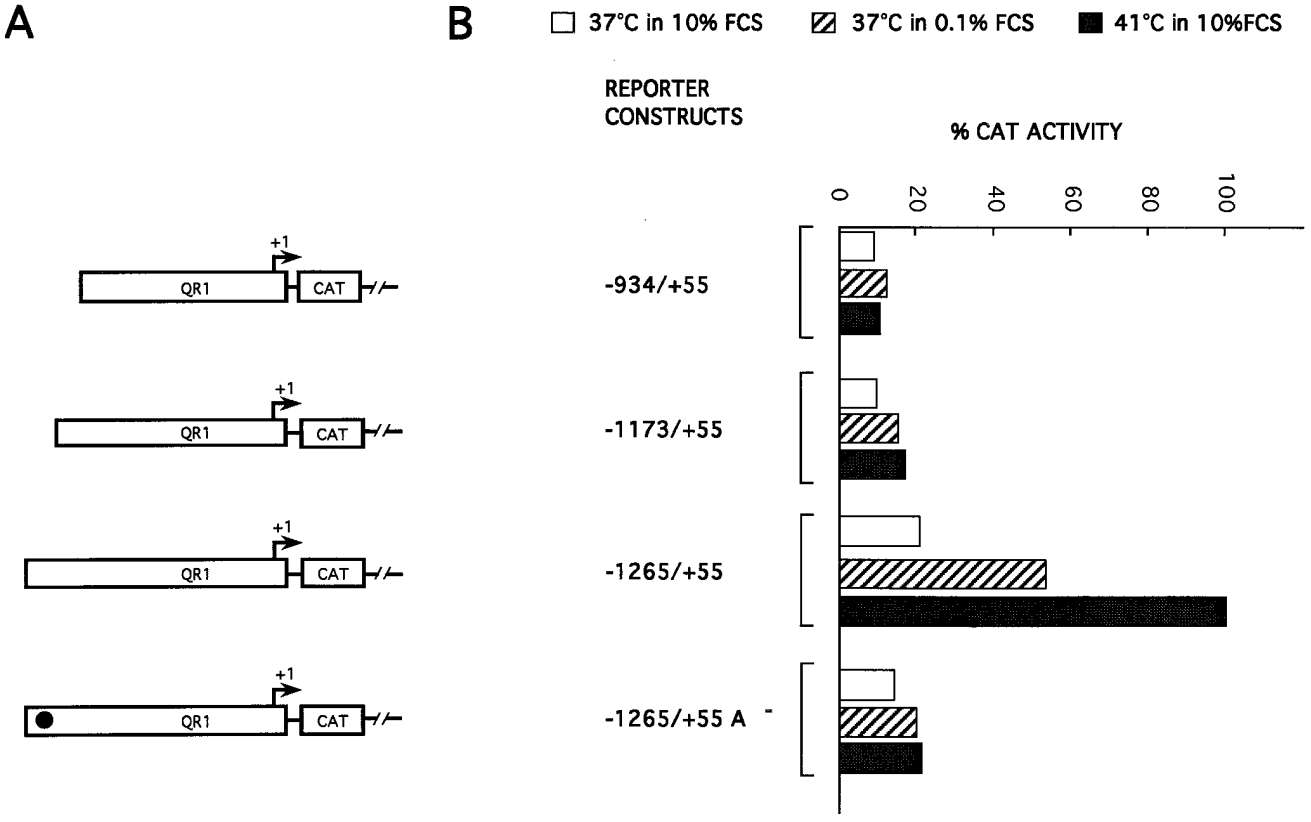


FIG. 3. The A box is involved in *QR1* transcriptional stimulation in response to cell quiescence. (A) Plasmids containing different deletions of the *QR1* 5′ flanking sequences up to position +55 with respect to the major transcription start site driving transcription of the CAT reporter gene were transfected into *ts*PA101-infected QNR cells along with a β-actin–*lacZ* plasmid. (B) Extracts from transfected cells maintained at 37°C in 10% FCS (open bars) or 0.1% FCS (hatched bars) and at 41°C in 10% FCS (solid bars) were tested for their CAT activities. CAT activity was measured by scintillation counting after adjustment for differences in β-galactosidase production and protein concentration. CAT activities are expressed as percentages of that of the −1265/+55 (WT) construct at 41°C. Each transfection was repeated in at least five independent experiments, with a 0.2- to 0.5-fold experimental variability for the stimulation. The constructs used in each transfection are indicated by their 5 and 3′ end extremities with respect to the major transcription initiation site of the *QR1* gene. The black circle indicates the presence of the mutation of the A box in the −1265/+55A⁻ construct.

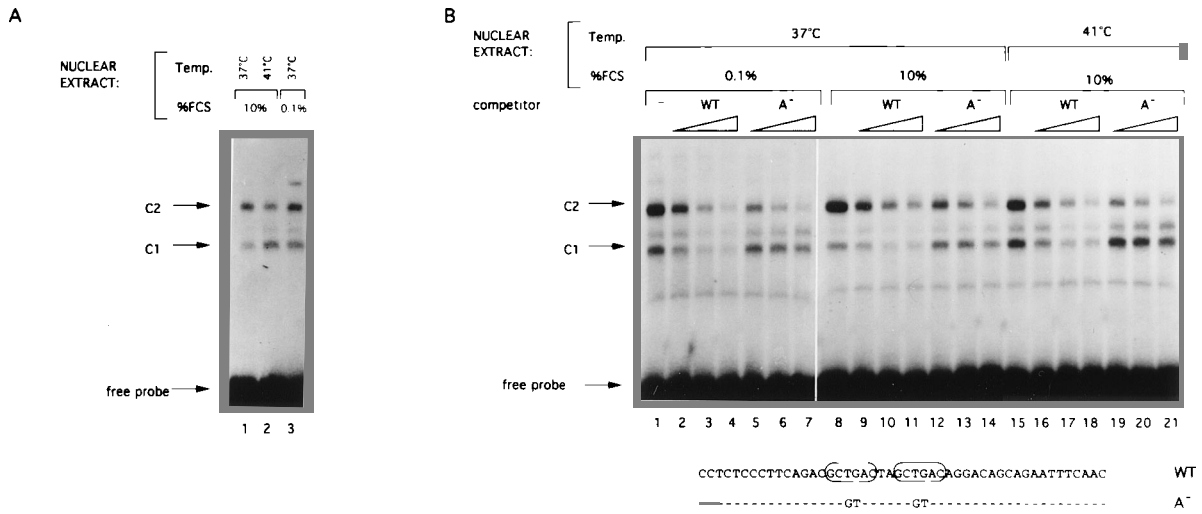


FIG. 4. Formation of the C1 complex increases upon growth arrest. (A) EMSA were performed with a ^{32}P-labeled kinase-treated double-stranded oligonucleotide spanning from bp −1208 to −1161 as a probe. Nuclear extracts were prepared from *ts*PA101-infected QNR cells grown at 37°C in 10% FCS (lane 1), at 41°C in 10% FCS (lane 2), and at 37°C in 0.1% FCS (lane 3). (B) EMSA were performed with nuclear extracts prepared from cells maintained at 37°C in 0.1% FCS (lanes 1 to 7), at 37°C in 10% FCS (lanes 8 to 14), or at 41°C in 10% FCS (lanes 15 to 21) in the absence of competitor DNA (lanes 1, 8, and 15) or in the presence of unlabeled WT oligonucleotide (lanes 2 to 4, 9 to 11, and 16 to 18) or unlabeled A$^-$ oligonucleotide mutated in both repeats (lanes 5 to 7, 12 to 14, and 19 to 21). The sequences of the A box (WT and A$^-$) are shown below the lanes. The two repeats are boxed. Temp., temperature.

Comparison of the levels of CAT activity directed by constructs containing bp −1173/+55 and bp −1265/+55 fragments of the *QR1* 5′ sequences indicated that an element(s) responding to cell quiescence was located in the region between positions −1265 and −1173 upstream from the transcription start site. The partial reactivation of *QR1* transcription upon serum starvation in comparison with that obtained by v-Src inactivation (41°C) is consistent with the results obtained by Northern blotting with steady-state RNAs. However, since *QR1* transcriptional stimulation in serum-starved cells reached about 50% of the levels obtained at 41°C, we cannot rule out the possibility that in cells expressing a functional v-Src protein, regulation of *QR1* expression could also involve a posttranscriptional control.

We previously reported that a v-*src*-responsive unit, designated the A box, is located between bp −1161 and −1208 upstream of the *QR1* transcription initiation site (63). The A box is composed of two repeats (GCTGACTAGCTGAC). Inversion of the TG in both repeats (−1265/+55A$^-$) abolishes the response of the *QR1* promoter to v-Src inactivation (63). We therefore tested whether the same mutation could affect quiescence-specific regulation of *QR1* transcription. We found that transcriptional activity of the −1265/+55A$^-$ construct was markedly reduced in serum-starved cells (0.1% FCS) at 37°C (Fig. 3) and reached the levels of the −1173/+55 construct, which does not contain the A box.

In conclusion, these results indicate that regulation of *QR1* expression by cell quiescence occurs, at least in part, at the transcriptional level. The A box, which is involved in transcriptional stimulation of the *QR1* gene upon v-Src inactivation (63) and during development (64), also mediates its stimulation upon growth arrest. However, inactivation of pp60$^{v\text{-}src}$ is necessary to completely release repression of *QR1* expression.

Formation of the C1 complex is increased upon growth arrest. In a previous report (63), we showed that the A box allows the formation of two protein-DNA complexes, C1 and C2. C1 is preferentially detected in nuclear extracts prepared from cells maintained at 41°C. Mutation of the two repeats (A$^-$) abolishes formation of this complex. In contrast, the C2 complex is more abundant in nuclear extracts from dividing cells at 37°C, and its binding to DNA is not affected by the A$^-$ mutation. Formation of the C1 complex is directly linked to transcriptional stimulation of the *QR1* gene upon v-Src inactivation and during NR development (64).

To biochemically characterize the transactivating factor(s) involved in quiescence-specific regulation at the A box, we performed electrophoretic mobility shift assays (EMSA) with nuclear extracts prepared from *ts*PA101-infected QNR cells maintained either at 41°C in 10% FCS or at 37°C in 10 or 0.1% FCS. A sequence spanning nucleotides −1208 to −1161 promoted the formation of two complexes, C1 and C2 (Fig. 4A). On the one hand, the C2 complex was detected at higher levels in nuclear extracts from cells expressing a functional pp60$^{v\text{-}src}$ (37°C) (Fig. 4A, lanes 1 and 3), independently of the proliferative state of the cells, since its level was comparable in extracts from either growth-arrested (0.1% FCS, lane 3) or proliferating (10% FCS, lane 1) cells at 37°C. However, formation of the C2 complex decreased upon v-*src* inactivation (Fig. 4A, lane 2). On the other hand, the C1 complex was more abundant in cells growth arrested by either serum starvation (Fig. 4A, lane 3) or pp60$^{v\text{-}src}$ inactivation (Fig. 4A, lane 2) than in growing cells (Fig. 4A, lane 1). We did not observe significant differences in the levels of C1 complex formation between extracts from cells maintained at 37°C in 0.1% FCS or at 41°C (Fig. 4A; compare lanes 3 and 2). An additional complex could be occasionally detected in extracts from cells maintained at 37°C in 0.1% FCS and seemed to be specific for these cells, since it was not detected in the other nuclear extracts. However, its formation was not reproducibly observed, since it was barely detected in the competition experiments (Fig. 4B, lane 1).

To verify that the complex induced in cells growth arrested by serum starvation corresponded to the C1 complex observed at 41°C, we used competition experiments (Fig. 4B). Nuclear extracts were incubated with the −1208/−1161 probe in the presence of increasing concentrations of unlabeled WT or double mutant (A$^-$) oligonucleotides. The WT oligonucleotide was able to compete equally well for C1 complex formation in extracts from growth-arrested cells (Fig. 4B, lanes 2 to 4 [37°C

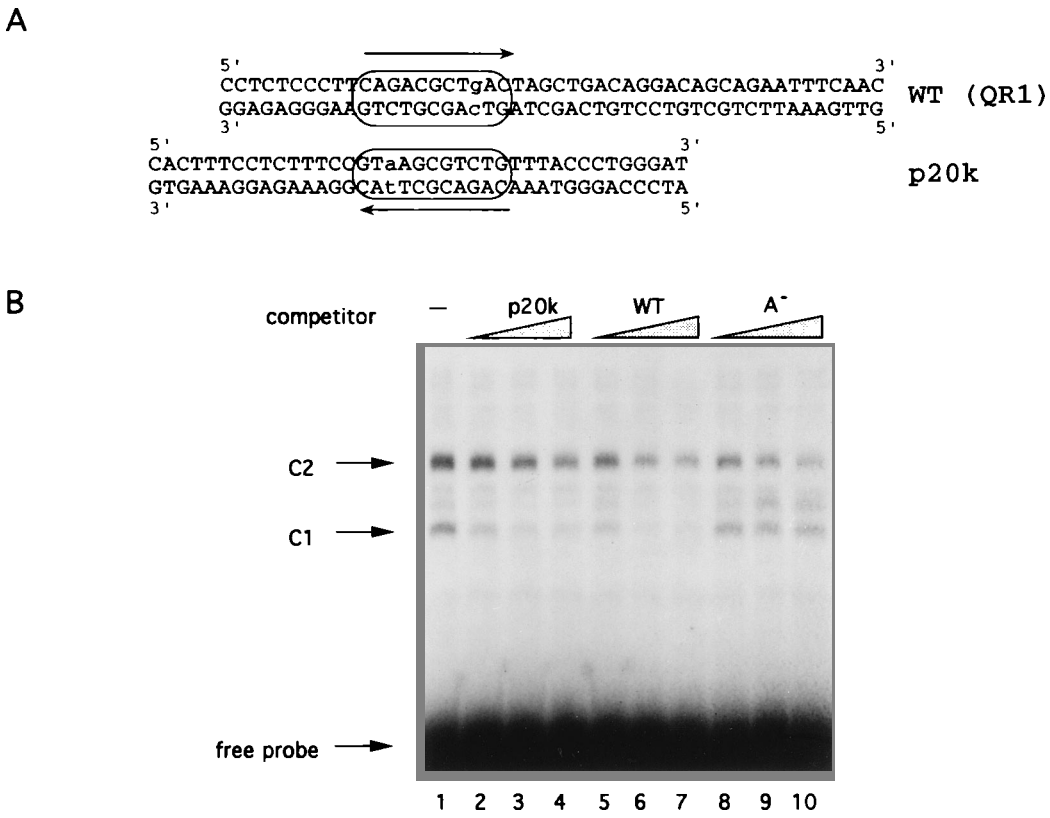


FIG. 5. C1 complex formation is prevented by an excess of unlabeled oligonucleotide derived from the QRU of the p20k gene. (A) Sequence comparison between the QRU of the p20k gene and the A box of the *QR1* gene. Sequence similarities are boxed. (B) EMSA experiments were performed with nuclear extracts prepared from *ts*PA101-infected QNR cells maintained at 37°C in 0.1% FCS. A standard binding reaction was performed with a ^{32}P-labeled *QR1* oligonucleotide (−1208 WT int) as a probe in the absence of competitor DNA (lane 1) or in the presence of increasing concentrations of an oligonucleotide containing the p20k QRU (lanes 2 to 4) or the *QR1* WT (lanes 5 to 7) or A$^-$ (lanes 8 to 10) oligonucleotide.

in 0.1% FCS] and lanes 16 to 18 [41°C]) and from proliferating cells (lanes 9 to 11 [37°C in 10% FCS]), whereas an excess of the A$^-$ competitor was unable to do so (Fig. 4B, lanes 5 to 7, 19 to 21, and 12 to 14, respectively). This confirmed that in growth-arrested cells expressing an active v-Src oncoprotein, the complex induced by cell quiescence is the C1 complex. Thus, formation of the C1 complex appears to be linked to the proliferative state of the cells.

In conclusion, our data suggest that growth arrest-specific transcriptional regulation of *QR1* gene expression is directly linked to C1 complex formation. Hence, C1 appears to respond to growth arrest both during NR development and in cells expressing the v-Src protein.

A QRU was recently identified in the 5′ upstream sequences of the p20k gene (57). This QRU (a 48-bp fragment) is able to confer growth arrest responsiveness to a heterologous promoter. However, the exact sequence in the 48 bp responsible for this regulation has not yet been determined. The first repeat of the *QR1* A box together with the 5 upstream nucleotides are partially homologous to a sequence contained in the QRU of the p20k promoter (Fig. 5A). To investigate whether the same, or a related, complex could be involved in the transcriptional activation of these two growth arrest-responsive genes, we used competition EMSA. Nuclear extracts from cells maintained at 37°C in 0.1% FCS were incubated with the −1208/−1161 probe, which contains the A box and the upstream conserved region, in the presence of an excess of unlabeled p20k QRU, *QR1* WT, or double mutant (A$^-$) oligonucleotide (Fig. 5B). The p20k oligonucleotide was able to compete for C1 complex formation in a dose-dependent manner, whereas formation of the C2 complex was only slightly affected (compare lanes 1 with lanes 2 to 4). The efficiency of the p20k oligonucleotide in competing for formation of the C1 complex was lower than that of the WT oligonucleotide (Fig. 5B, lanes 2 to 4 and lanes 5 to 7, respectively) but much higher than that of the A$^-$ mutant oligonucleotide (Fig. 5B, lanes 8 to 10). These data suggest that the C1 complex is specifically induced by cell quiescence and that it may be involved in the transcriptional regulation of other growth arrest-specific genes.

The C1 complex involves a protein related to the Maf family. The repeated element in the A box also displays similarity with the recently identified DNA binding site of the Maf family proteins (TGCTGA[C/G]TCAGCA) (47, 50). One repeat of the A box represents an almost perfect half of the palindromic DNA sequence to which Maf proteins bind. To test the possibility that a protein related to this family could participate in formation of one of the characterized complexes, EMSA experiments using nuclear extracts prepared from quiescent cells maintained at 37°C in 0.1% FCS (Fig. 6A) or at 41°C (Fig. 6B) were done in the presence of immune sera raised against different Maf proteins.

The formation of both C1 and C2 complexes was increased by addition of bovine serum albumin (BSA) (compare lanes 1 and 2 in Fig. 6). The addition of nonimmune serum did not affect the formation of these two complexes, except that it accelerated migration of the C1 complex (Fig. 6, lanes 6). This

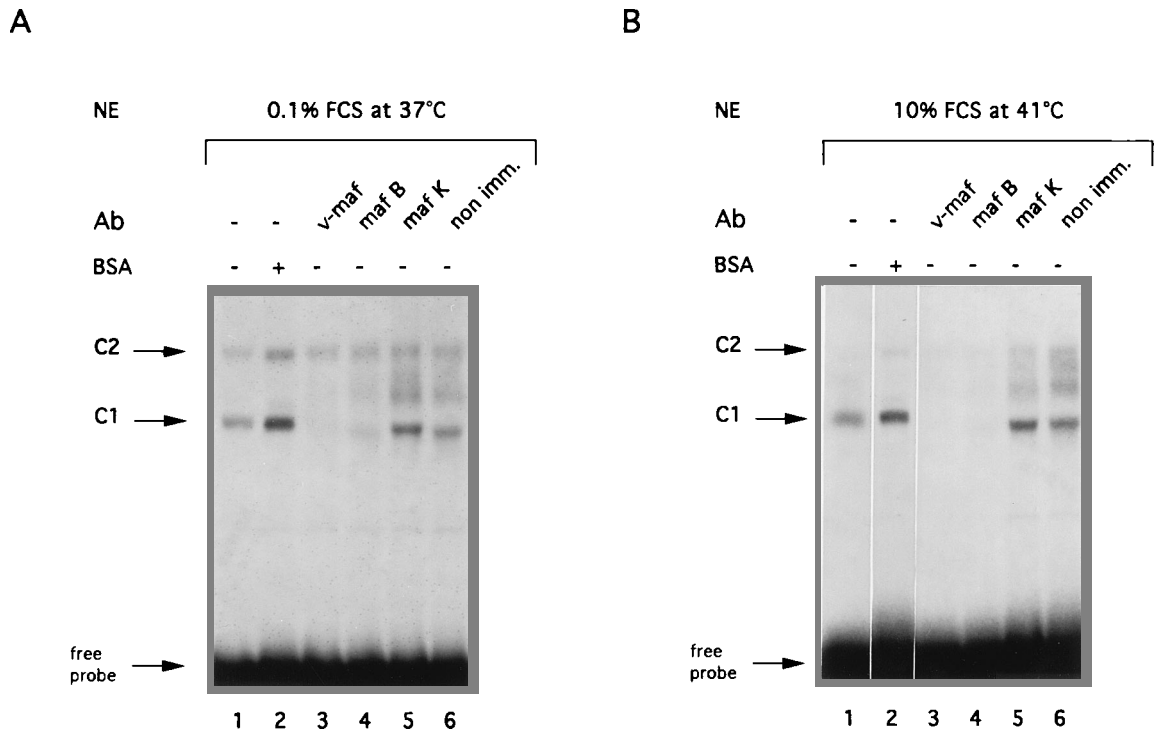


FIG. 6. Formation of the C1 complex is inhibited by addition of v-Maf and MafB antisera. EMSA were performed with a ^{32}P-labeled kinase-treated double-stranded oligonucleotide spanning from bp −1208 to −1161 as a probe. Nuclear extracts (NE) prepared from *ts*PA101-infected QNR cells maintained at 37°C in 0.1% FCS (A) or at 41°C in 10% FCS (B) were preincubated for 15 min alone (lanes 1) or in the presence of BSA (lanes 2) or anti-v-Maf (lanes 3), anti-MafB (lanes 4), anti-MafK (lanes 5), or nonimmune (non imm.) (lanes 6) serum. Ab, antibody.

nonspecific effect was observed with various antisera (data not shown). We verified that this accelerated complex corresponded to the C1 complex, since its formation was inhibited by an excess of unlabeled *QR1* WT oligonucleotide but not by the mutant A$^-$ oligonucleotide (data not shown). An immune serum directed against MafK, which is one of the small members of the Maf protein family lacking the transactivation domain, had an effect similar to that of the nonimmune serum (Fig. 6, lanes 5). In contrast, both the anti-v-Maf and anti-MafB sera were able to inhibit formation of the C1 complex (Fig. 6, lanes 3 and 4). Because of the cross-reactivity between v-Maf and MafB immune sera, due to the high degree of similarity of these proteins (48), our experiments did not allow further identification of the protein, antigenically related to Maf, that is involved in C1 complex formation. The effects of the different antisera on C1 complex formation were identical whether nuclear extracts were prepared from growth-arrested cells maintained at 41°C (Fig. 6B) or in 0.1% FCS at 37°C (Fig. 6A). We did not detect any effect of these antisera on formation of the C2 complex.

To test the specificities of the sera, we analyzed by Western blotting (immunoblotting) nuclear extracts prepared from *ts*PA101-infected QNR cells maintained at 41°C and probed with either anti-v-Maf or anti-MafB serum (Fig. 7A). The anti-v-Maf serum recognized three proteins (Fig. 7A, lane 2). Only a 43-kDa polypeptide seemed to be specific, since it was not revealed after adsorption of the serum with the antigen, whereas the upper bands were still observed under these conditions (Fig. 7A; compare lanes 2 and 3). The MafB antiserum also detected a 43-kDa protein as well as several other products (Fig. 7A, lane 1). However, only recognition of the 43- and 39-kDa polypeptides was prevented by serum preabsorption (data not shown). Interestingly, only the 43-kDa protein was recognized by both sera. Neither the MafK nor the nonimmune serum recognized this 43-kDa protein (data not shown). In addition, we found that the level of a 43-kDa polypeptide was increased in *ts*PA101-infected NR cells transfected with an expression vector carrying the c-*maf*II gene compared with that in cells transfected with the pEF vector alone (Fig. 7B, lanes 1 and 2) and that this protein was specifically recognized by the v-Maf antiserum. These results confirm the ability of the v-Maf antiserum to recognize overexpressed Maf proteins and suggest that the 43-kDa protein present in nuclear extracts of *ts*PA101-infected cells is a protein related to this family. Therefore, inhibition of C1 complex formation by the anti-v-Maf and anti-MafB sera is likely to be correlated with the abilities of both antisera to interact with this 43-kDa protein.

We also compared the levels of the 43-kDa Maf-related protein in nuclear extracts prepared from cells maintained at 37°C in 10% (Fig. 7C, lane 1) or 0.1% (Fig. 7C, lane 3) FCS or at 41°C in 10% FCS (Fig. 7C, lane 2) by Western blot analysis with the anti-v-Maf serum. The 43-kDa protein was detected in all three nuclear extracts but at a lower level in proliferating cells (lane 1) than in growth-arrested cells (lanes 2 and 3). Thus, this apparent increase of the 43-kDa protein appears to correlate with that of C1 complex formation.

We next tested whether a purified bacterially expressed v-Maf fusion protein was capable of binding the A box. To do this, we performed EMSA experiments using the following labeled oligonucleotides as probes: oligonucleotide 1 containing a Maf consensus site (47), the *QR1* WT oligonucleotide, and their corresponding mutants (oligonucleotides 23 and A$^-$, respectively) (Fig. 8). We found that oligonucleotide 1 formed two specific complexes with the v-Maf protein (Fig. 8, lane 3),

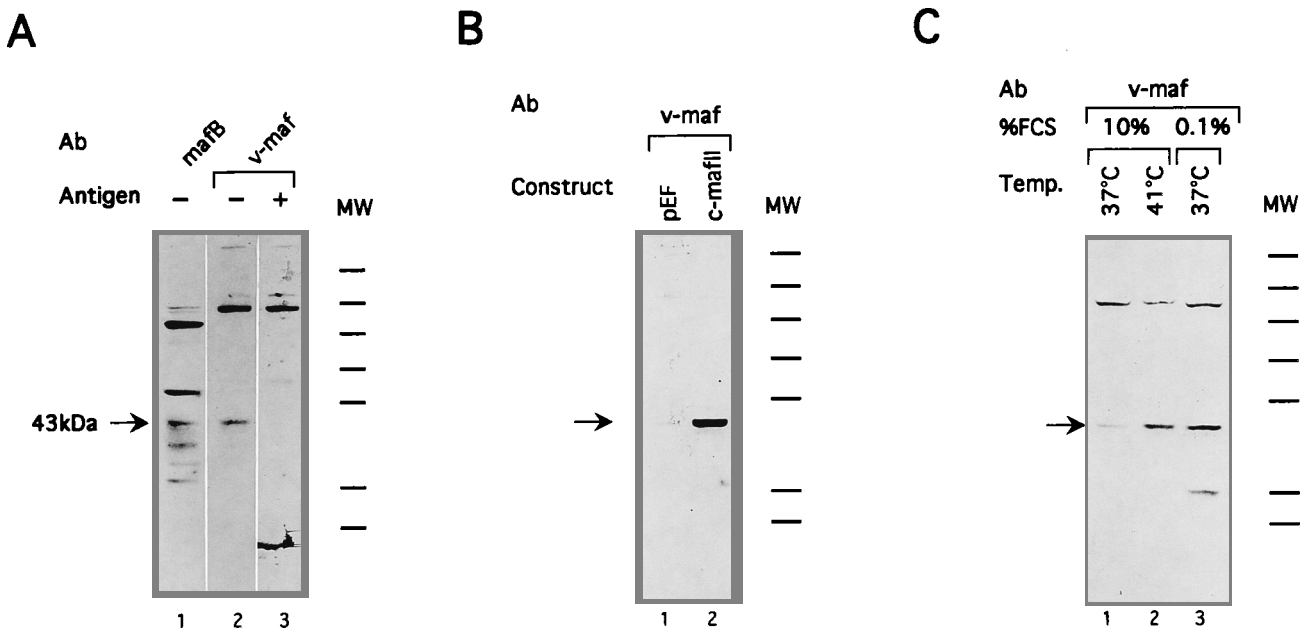


FIG. 7. A 43-kDa protein is specifically recognized by v-Maf and MafB antisera in *ts*PA101-infected QNR cells. (A) Nuclear extracts (37.5 μg) prepared from cells maintained at 41°C in 10% FCS were resolved by SDS-PAGE and blotted on nitrocellulose filters. Membranes were probed with anti-MafB serum (lane 1), anti-v-Maf serum (lane 2), or anti-v-Maf serum preincubated with the antigen (lane 3). (B) Total cell extracts (95 μg) from *ts*PA101-infected QNR cells maintained at 37°C in 10% FCS and transfected with 10 μg of the pEF control vector (lane 1) or c-*maf*II expression vector (lane 2) were analyzed by Western blotting with the v-Maf antiserum. (C) Nuclear extracts (50 μg) prepared from cells maintained at 37°C in 10% FCS (lane 1) or 0.1% FCS (lane 3) or at 41°C in 10% FCS (lane 2) were analyzed by Western blotting with the v-Maf antiserum. Molecular mass markers (MW) correspond to, from the top, 195, 112, 84, 63, 52.5, 35, and 32 kDa. Ab, antibody.

since they were not observed with the mutant oligonucleotide 23 (lane 4). The labeled *QR1* WT oligonucleotide also formed two complexes with purified v-Maf protein, although the more slowly migrating complex was detectable after prolonged exposure (Fig. 8, lane 5). Formation of these two complexes was dependent on the integrity of the A box, since the *QR1* A^- oligonucleotide was no longer capable of allowing their formation (lane 6). We confirmed that both complexes on the Maf and *QR1* probes contained the v-Maf protein by preincubation with either preimmune (Fig. 8, lanes 7 and 9) or anti-v-Maf (lanes 8 and 10) serum. Only the anti-v-Maf serum was able to inhibit complex formation. The presence of two complexes is likely to result from formation of monomeric and multimeric complexes (83).

To further assess the presence of a Maf-related protein(s) in the C1 complex, we tested oligonucleotides which differ in their abilities to bind Maf homo- and heterodimers (47) for their capacities to compete for C1 complex formation in an EMSA. An oligonucleotide (no. 29) containing a Maf nonpalindromic sequence, which was previously shown to bind a Maf/Jun heterodimer efficiently and a Maf homodimer with a lower efficiency but not Jun homodimers or Jun/Fos heterodimers, was able to compete for C1 complex formation at a level comparable to that found for the WT oligonucleotide (Fig. 9). Palindromic Maf consensus sites (oligonucleotides 1 and 2) were also able to compete, although not as efficiently as the WT oligonucleotide or oligonucleotide 29. In contrast, oligonucleotide 23, which corresponds to a consensus site for the AP-1 complex and does not bind Maf proteins, did not compete, like an unrelated oligonucleotide (NS in Fig. 9). Interestingly, sequence comparison of oligonucleotides 1, 2, 23, and 29 and the WT oligonucleotide within the region spanning the A box first repeat suggests that the presence of the GC nucleotides (boxed in Fig. 9) previously shown to be required for binding of Maf proteins (50) also correlates with the capacity to compete for C1 complex formation. Taken together, the antibody inhibition, the oligonucleotide competition, and the capacity of a Maf protein to bind the A box strongly suggest that a Maf-related protein(s) is involved in formation of the C1 complex, likely in the form of an heteromer.

Transcription of the *QR1* gene is stimulated by c-MafI, c-MafII, and MafB and is inhibited by MafK. To test the possibility that a Maf protein could participate in stimulating *QR1* transcription through the A box, we cotransfected vectors encoding different members of this family (40, 44) together with *QR1* reporter plasmids in *ts*PA101-infected QNR cells maintained at 41°C (Fig. 10). The experiments were performed at 41°C to ensure the formation of potential heterodimers with proteins specifically present at this temperature and to avoid posttranslational modifications that could alter the transcriptional activity of Maf proteins at 37°C in the presence of an active $pp60^{v\text{-}src}$. Moreover, as mentioned above, we did not observe differences in C1 complex formation when using extracts of quiescent cells maintained in 0.1% FCS at 37°C or in 10% FCS at 41°C.

We observed a four- to eightfold transcriptional stimulation of the −1265/+55 CAT construct upon expression of the c-MafI, c-MafII, and MafB proteins with respect to that of the pEF vector alone (Fig. 10). Transcriptional stimulation by the c-MafI and c-MafII proteins was markedly reduced upon mutation of the A box (−1265/+55A^-) and was abolished when the −1265/+55A^- construct was cotransfected with the *maf*B expression plasmid, relative to that for the pEF expression vector alone. In contrast, no transactivation was observed on the CAT5 control reporter plasmid upon expression of the different Maf proteins (Fig. 10). A twofold stimulation was measured on a reporter construct containing sequences up to bp −345 of the *QR1* promoter upon expression of the c-*maf*I and c-*maf*II genes, whereas cotransfection of the *maf*B and *maf*K genes had no effect on the transcriptional activity of this

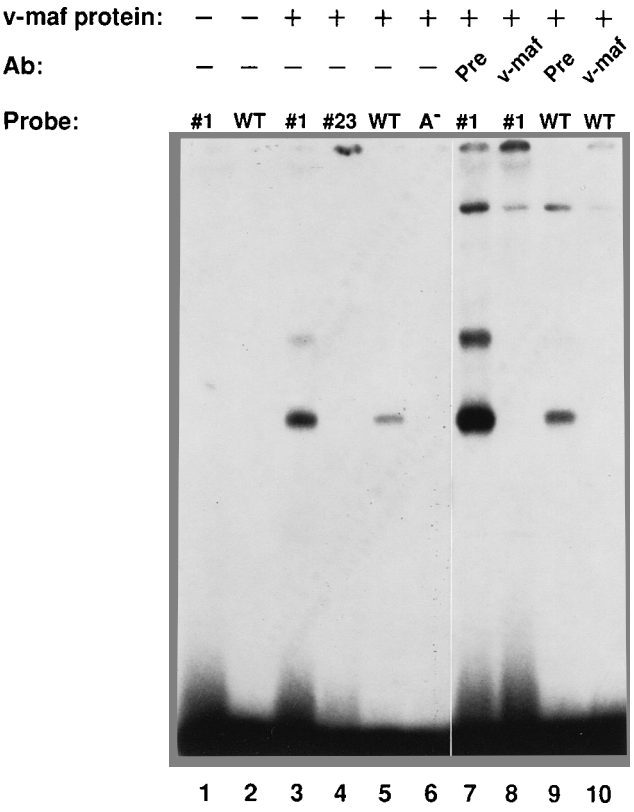
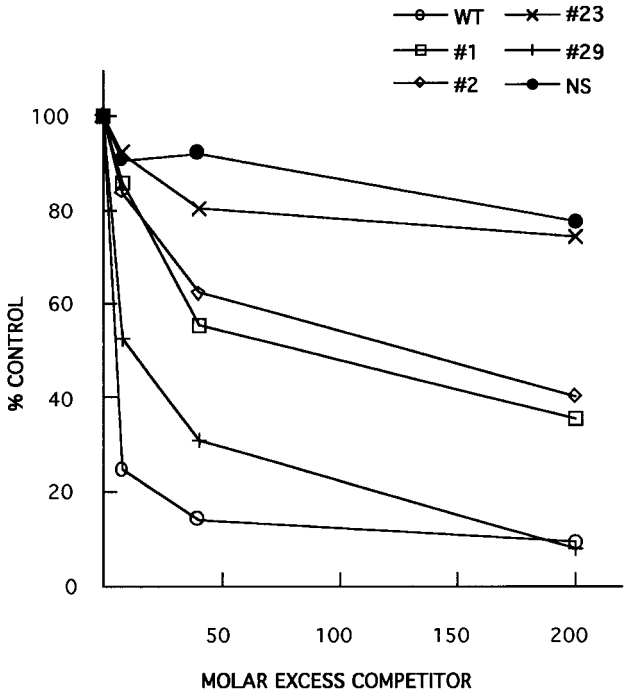


FIG. 8. A bacterially expressed v-Maf protein binds to the A box. EMSA were performed with 50 ng of purified bacterially expressed maltose-binding protein–v-Maf fusion protein and the following ^{32}P-labeled kinase-treated double-stranded oligonucleotides as probes: oligonucleotide 1 (Maf consensus site) (lanes 1, 3, 7, and 8), oligonucleotide 23 (Maf consensus site mutant) (lane 4), and *QR1* WT (lanes 2, 5, 9, and 10) and A$^-$ (lane 6) oligonucleotides. In lanes 7 to 10 the fusion protein was preincubated for 15 min in the presence of preimmune (Pre) (lanes 7 and 9) or anti-v-Maf (lanes 8 and 10) serum. No protein was added in lanes 1 and 2. Ab, antibody.



WT	CCTCTCCTTCAGACGCTGACTAGCTGAC
#1	TCGAGCTCGGAATTGCTGACTCAGCATTACTC
#2	TCGAGCTCGGAATTGCTGACGTCAGCATTACTC
#23	TCGAGCTCGGAATCAATGACTCATTGTTACTC
#29	TCGAGCTCGGAATTGCCGACTCATTGTTACTC

FIG. 9. C1 complex formation is prevented by addition of unlabeled oligonucleotides containing Maf binding sites. Competition-DNA EMSA were performed with nuclear extracts prepared from *ts*PA101-infected QNR cells maintained at 41°C in 10% FCS. A standard binding reaction was performed for 45 min with a ^{32}P-labeled *QR1* WT oligonucleotide (−1208/−1161) as a probe in the absence of competitor DNA or in the presence of increasing concentrations of oligonucleotides containing *QR1* WT, Maf homo- and heterodimer consensus sites (no. 1, 2, and 29), Maf mutated site (no. 23), or nonspecific sequences (NS). The sequences of the oligonucleotides used as competitors are shown at the bottom. Nucleotides identical to the perfect Maf palindromic consensus site are underlined. The amount of radioactive −1208 WT int probe bound in the C1 complex in the absence of competitor DNA was taken as 100%. The percentages of the control value in the presence of different competitor oligonucleotides are reported on the vertical axis, and the molar excess of competitor DNA is reported on the horizontal axis.

construct. These results suggest that the integrity of the A box is necessary for transcriptional activation of *QR1* expression by the MafB protein. However, transcriptional stimulation of the *QR1* −1265/+55 construct upon expression of c-*maf*I and c-*maf*II is not completely abolished by mutation in the A box. Hence, these two proteins are able to transactivate *QR1* gene expression by acting at the level of the A box, but they could also interact elsewhere in a more proximal region(s) of the promoter.

In contrast, cotransfection of the *maf*K gene repressed transcription of the −1265/+55 reporter plasmid to about 30%, of that of the pEF vector. This transcriptional repression was almost completely abolished by a mutation in the A box (Fig. 10; compare reporter plasmids −1265/+55 and −1265/+55A$^-$ in the presence of the *maf*K expression plasmid with the same reporter constructs cotransfected with the pEF expression vector alone). Cotransfection of increasing concentrations of the *maf*K expression plasmid in the presence of the c-MafII protein showed that MafK was able to inhibit *QR1* transcriptional stimulation mediated by the c-*maf* gene (data not shown). We conclude that different members of the Maf family are able to stimulate or repress *QR1* promoter activity, possibly by interacting with the A box.

To test whether the A box was sufficient to mediate the effects of the Maf proteins, we used a reporter plasmid containing the A box multimerized four times (4×WT) upstream of the heterologous TK promoter (Table 1). No variations were observed on the control heterologous promoter (TK10) upon cotransfection of different members of the *maf* family (c-*maf*I, c-*maf*II, *maf*B, and *maf*K). In contrast, 275- and 60-fold stimulations of transcription of the reporter gene containing the multimerized A box (4×WT) were observed upon expression of c-*maf*I (or c-*maf*II) and *maf*B cDNAs, respectively. Transcriptional stimulation was almost completely abolished when the mutated concatemerized A box was used as a reporter plasmid (4×A$^-$). A repression to 50% of the activity of the 4×WT but not the 4×A$^-$ construct was reproducibly observed upon cotransfection of the *maf*K expression plasmid. This inhibition seemed, therefore, to also be dependent on the

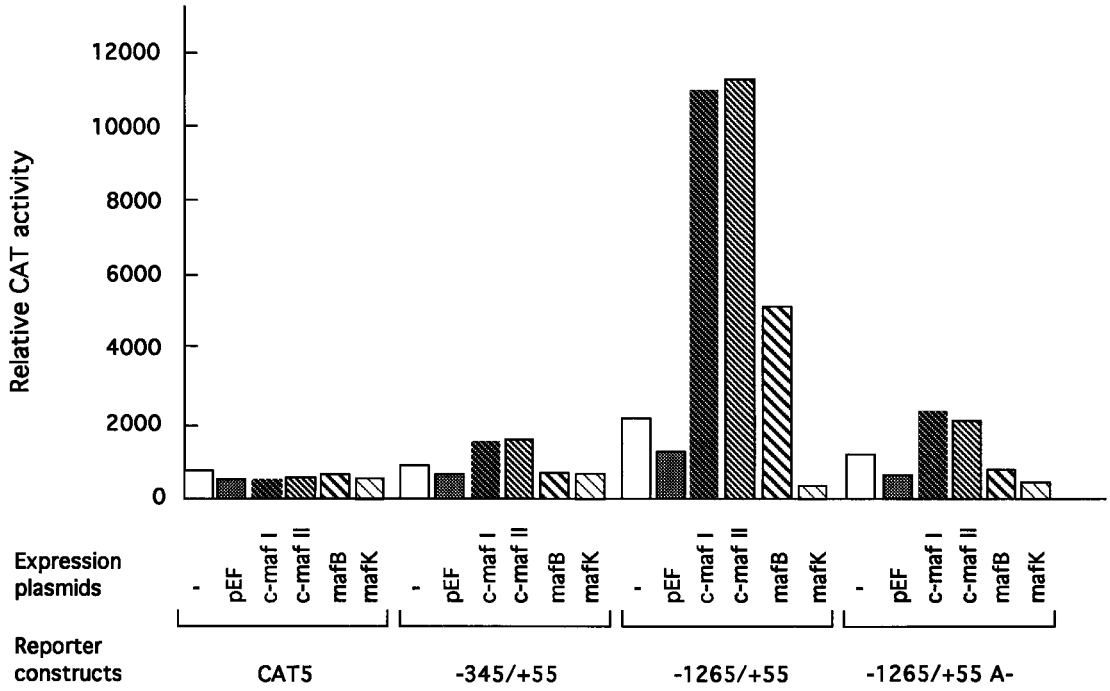


FIG. 10. Stimulation of *QR1* transcription by the c-MafI, c-MafII, and MafB proteins and requirement for the integrity of the A box. *ts*PA101-infected QNR cells maintained at 41°C in 10% FCS were transfected with 10 μg of the CAT5 reporter plasmid or with one of the following CAT5/*QR1* reporter constructs: −345/+55, not containing the A box; −1265/+55, containing the A box; and −1265/+55A⁻, containing a mutated A box. Cells were cotransfected with 3 μg of carrier DNA (open bars) or the pEF expression vector alone or with the c-*maf*I, c-*maf*II, *maf*B, or *maf*K gene expression plasmid. CAT activity was assayed after normalization for β-galactosidase activity and protein concentration. CAT activity measured by scintillation counting was plotted and is expressed as converted counts per hour. Results are averages from three to five independent experiments, with a 0.2- to 0.4-fold experimental variability for the stimulation.

integrity of the A box. Taken together, our data indicate that the A box is sufficient to mediate stimulation of *QR1* transcription by proteins of the Maf family.

DISCUSSION

Cell proliferation and terminal differentiation are often mutually exclusive processes. The mechanisms which lead to the loss of proliferative capacity and terminal differentiation remain poorly understood. The temporal and spatial patterns of expression of *QR1*, a retina-specific gene, suggest that it may play a role at the transition from the undifferentiated proliferating precursor cell stage to the differentiated postmitotic cell stage. In this study, we investigated the role of cell division in downregulation of *QR1* expression by v-Src. We reported that transcription of the *QR1* gene is stimulated upon NR cell growth arrest. This response to cell quiescence correlates with the induction of C1 nucleoprotein-DNA complex formation on the A box. We also show that this complex appears to involve a factor(s) related to the Maf family and that Maf proteins are capable of binding the A box and stimulating transcription of the *QR1* gene. Mutations of the A box which abolish C1 complex formation, as well as v-Maf protein binding to the A box, also prevent stimulation of *QR1* transcription by Maf proteins.

TABLE 1. The multimerized A box is sufficient to confer to a heterologous promoter the capacity to be transactivated by Maf proteins

Expression plasmid	Relative CAT activity[a] with reporter construct:			Fold stimulation[b] with reporter construct:		
	TK10	4×WT	4×A⁻	TK10	4×WT	4×A⁻
None	1.1	1.4	1.1	1.1	1.0	1.1
pEF	1.0	1.4	1.0			
c-*maf*I	1.0	385.0	2.3	1.0	275.0	2.3
c-*maf*II	1.0	390.0	1.6	1.0	278.6	1.6
*maf*B	0.9	81.0	0.8	0.9	57.9	0.8
*maf*K	1.1	0.7	1.6	1.1	0.5	1.6

[a] Expressed as a percentage of converted counts per hour.
[b] Expressed as a ratio of the CAT activity in the presence of the *maf* expression plasmid to that of the control pEF expression plasmid alone.

How does v-*src* prevent expression of a differentiation-specific gene in the retina? In contrast to other cell types infected by Rous sarcoma virus (41, 72, 81), QNR cells expressing an active v-*src* gene product remain dependent on growth factors present in the serum. This dependence is not correlated with the morphology-transforming capacities of the v-Src protein or with its levels of tyrosine kinase activity, since identical results were obtained with NR cells infected by either *ts*NY68 (30, 66) or *ts*PA101 virus. The cooperation between serum and v-Src in the induction of QNR cell division allowed us to study how pp60$^{v\text{-}src}$ acts to repress expression of differentiation-specific genes.

We have shown that *QR1* expression is partially restored in quiescent *ts*PA101-infected QNR cells expressing an active v-Src oncoprotein (37°C in a low serum concentration). Steady-state mRNA levels were relatively low in cells growth arrested by serum starvation compared with those in cells rendered quiescent upon v-Src inactivation at 41°C. In contrast, the transcriptional activity (CAT activity) of the −1265 *QR1* upstream sequences in cells maintained at 37°C in 0.1% FCS appears to be reduced by only 50% with respect to the level detected at 41°C. It is therefore possible that, in addition to a transcriptional control, a posttranscriptional mechanism could

be involved in the regulation of *QR1* gene expression by pp60$^{v\text{-}src}$. Control of RNA stability appears to be independent of the growth state of the cells, whereas transcriptional stimulation upon v-Src inactivation is controlled in a large part by growth arrest. However, the fact that transcriptional stimulation is higher upon v-Src inactivation suggests that an additional effect of pp60$^{v\text{-}src}$, independent of cell growth, is involved in repression of *QR1* transcription. We previously showed that pp60$^{v\text{-}src}$ represses transcription of the *QR1* gene in part by inhibiting formation of the C1 complex. This complex is induced at comparable levels by growth arrest upon either serum starvation or v-Src inactivation, suggesting that formation of the C1 complex is regulated primarily by growth arrest signals. In contrast, a second complex, C2, is induced upon expression of an active v-Src oncoprotein independently of cell growth. It is present at similar levels in cells maintained at 37°C independently of serum concentration and decreases upon pp60$^{v\text{-}src}$ inactivation at 41°C. Our unpublished data suggest that the C1 and C2 complexes require overlapping sequences to bind DNA. Thus, the C2 complex could represent a putative repressor that would prevent formation of the C1 complex by competing for overlapping sequences. The ratio between these two complexes on DNA may then determine the relative levels of transcriptional stimulation or repression. In conclusion, downregulation of *QR1* expression is the result of two events induced by v-Src, one dependent and the other independent of the proliferative state of the cells.

It is interesting that the C1 complex is also involved in transcriptional stimulation of the *QR1* gene during NR development and that the C2 complex is detected during early embryogenesis and in adult retinas when the *QR1* gene is not expressed (64). Thus, C1 correlates with growth arrest and *QR1* expression during NR differentiation, whereas C2 appears to correlate with repression of *QR1* transcription.

Our observations raise the interesting possibility that pp60$^{v\text{-}src}$ could reverse established differentiation processes on the one hand by perturbing cell growth and on the other hand by inhibiting expression of development- and tissue-specific genes. A mechanism common to several cell types may be responsible for altering cell growth and would involve the induction of immediate-early genes by targeting ubiquitously expressed transcription factors (5, 9, 20, 23, 35, 37, 67, 70, 82). A second pathway would negatively regulate differentiation-specific genes by targeting developmentally controlled transcription factors. Our results are in agreement with those of studies on muscle cell differentiation upon expression of pp60$^{v\text{-}src}$. Interestingly, in those cells a tight relationship between tissue-specific transcription factors and growth control has been demonstrated (31, 38, 39).

The C1 complex is induced upon NR cell growth arrest. Little is known about how transcriptional control by growth arrest occurs (22, 57). The expression of most of the growth arrest-specific genes isolated thus far is regulated at the post-transcriptional level (15, 17). In this study, we have shown that *QR1* transcription partially responds to cell quiescence. Therefore, the *QR1* gene can also be considered a growth arrest-responsive gene. We showed that growth arrest control of *QR1* transcription is mediated by the A box. Thus, the A box corresponds to a QRU in the 5′ upstream regulatory sequences of the *QR1* gene. The C1 complex, which is involved in v-*src* regulation, is also induced by growth arrest. Moreover, the binding of the C1 complex to DNA is inhibited by an oligonucleotide derived from the QRU of the growth arrest-specific p20k gene. Hence, a common factor(s) could be involved in the regulation of these two quiescence-specific genes, despite the tissue-specific expression of the *QR1* gene. Indeed, the C1 complex appears to be present in cell types other than retinal cells, although at lower levels (64, 66). Thus, as for cell division, a general pathway could be implicated in entering the G_0 phase, even in specialized cell types. If this is the case, cell-specific expression of the *QR1* gene would not be conferred by the A box but by an additional regulatory element(s). In support of this possibility, we have recently characterized a second *cis*-acting element, called the B box, which is involved in transcriptional regulation of the *QR1* gene during development and is able to bind neuro- and stage-specific factors of the POU family of transcriptional regulators (64). This regulatory element could be involved in restricting *QR1* transcription to NR cells. The integration of distinct cellular events through different regulatory elements, such as the A and B boxes, could explain the tightly regulated expression of the *QR1* gene.

The C1 complex contains a Maf-related protein(s). Since the C1 complex appears to play a key role in growth arrest during both retinal development and regulation by pp60$^{v\text{-}src}$, we searched for a factor(s) that could be involved in this complex. Our data, based on the use of different antisera against Maf proteins and competition with Maf consensus oligonucleotides in EMSA, suggest that a protein(s) related to the Maf family is involved in forming the C1 complex. Moreover, a v-Maf bacterially expressed protein can bind to the A box. By cotransfection with vectors expressing different Maf proteins, we showed that both c-MafI and -II and the MafB proteins stimulate *QR1* transcription through the A box. Mutation of the same nucleotides that abolish C1 binding also abolishes transcription of the *QR1* gene as well as binding of the v-Maf protein to the A box and stimulation of *QR1* transcription by Maf proteins. This is the first indication that a Maf-related protein(s) may play a role in transcriptional stimulation of a target gene in the NR. Maf proteins have been shown to form homo- as well as heterodimers with members of the leucine zipper family (4, 40, 44, 45, 47, 51). Distinct combinations of these factors exhibit different affinities and specificities for DNA (45, 47, 50, 52). Our results suggest that the C1 complex is not formed by a Maf homodimer, since competition for C1 complex formation was more efficient with oligonucleotides showing high affinities for Maf heterodimers than with oligonucleotides containing perfect palindromic sites for Maf homodimers. Putative natural binding sites for Maf proteins containing a transactivation domain, other than the *QR1* site, were recently identified in two genes. On the one hand, the *maf* proto-oncogene was reported to stimulate transcription of the *L7* gene (52). On the other hand, it was shown that a new member of the Maf family stimulates transcription of the α-crystallin gene and that Maf proteins bind efficiently to a site in the promoter of this gene (83). Interestingly, both the *L7* and α-crystallin Maf recognition sites have strong similarities to a half site of the palindromic Maf consensus sequence (MARE), as in the *QR1* gene. Thus, several lines of evidence indicate the presence of nonpalindromic sites for Maf proteins in their natural target sequences. As suggested by Kerppola and Curran (50), the DNA binding specificities of the Maf heterodimeric complexes are consistent with a combinatorial determination of DNA binding specificity through a mechanism in which each subunit in the complex determines the DNA binding specificity for one half site independently of its partner. Our results are consistent with the possibility that a Maf-related protein would bind to a half site in the *QR1* A box first repeat and that a distinct partner would bind to the adjacent sequence.

We also showed that the MafK protein inhibits transcription of both the −1265/+55 and the 4×WT reporter constructs. This repression seems to require integrity of the A box. Thus,

members of the Maf family lacking the transactivation domain might play a role in repression of *QR1* transcription.

The *maf* genes were initially identified as cellular homologs of the v-*maf* oncogene and shown to express transforming properties when overexpressed (29, 44, 46). The role of Maf-related proteins in cell quiescence could be envisaged in different ways. It is possible that a yet-unidentified member(s) of this family could act as a negative regulator of cell growth, as reported for JunD (62). Alternatively, participation of a known Maf protein in this process could also result from posttranslational modifications affecting its DNA binding, heterodimerizing, or transactivating properties. Finally, we cannot exclude the possibility that cell growth arrest would modulate the activity of another component(s) in the C1 complex. In this report, we showed that the antisera used to inhibit C1 complex formation specifically recognize a 43-kDa protein in *ts*PA101-infected NR cells. The apparent level of this protein is increased in nondividing cells, in correlation with that of the C1 complex. This Maf-related protein, which remains to be characterized, is therefore likely to play a role in regulating *QR1* transcription.

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