Maf and Jun Nuclear Oncoproteins Share Downstream Target Genes for Inducing Cell Transformation*

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The Maf oncoprotein is a basic leucine zipper (bZip)-bearing transcriptional activator that recognizes the Maf recognition element (MARE) DNA sequence. In this study, we investigated the role of Maf's transactivation function in cell transformation. Replacement of the conserved amino terminus transactivator domain of Maf by a heterologous and stronger transactivator domain (the acidic transactivator domain of VP16) resulted in enhanced transformation of chicken embryo fibroblast cells. In contrast, the fusing of a transcriptional repressor domain (Sin3 interaction domain of Mxi1) with the whole Maf protein masked the transactivator function of Maf, which in turn inhibited its transforming activity. Furthermore, the leucine zipper domain of Maf, which defines its dimer-forming specificity, was exchangeable with that of GCN4 yeast protein in terms of its transactivating and cell transforming activities. Thus, heterodimer formation with other bZip factors is not required for Maf's ability to transform. These results together suggest that transactivation through MARE is necessary for Maf-induced transformation and that there exist downstream target gene(s) for transformation. Since the MARE sequence overlaps with the recognition element of another bZip oncoprotein, Jun, we assessed whether Jun and Maf induce cell transformation through activating the same genes. We thus constructed a mutated version of Jun that has a GCN4 leucine zipper and lacks the transactivator domain. This mutant repressed the cell transformation not only by Jun but also by Maf. Thus, Maf and Jun share downstream target gene(s) that are involved in cell transformation.

The v-maf oncogene was originally identified in the genome of the acute transforming avian retrovirus AS42. It induces musculosaponeurotic fibrosarcoma in chickens and transforms primary chicken embryo fibroblast (CEF)§ cells (1). Analysis of the genome structures of the AS42 provirus and its cellular counterpart c-maf revealed that v-Maf does not differ structurally from c-Maf except that it fused to viral Gag protein at the initiator methionine residue of c-Maf (2). Supporting this finding is the observation that c-maf efficiently transforms CEF cells when it is expressed under the control of the retroviral promoter (3). Recently, c-maf was found to be overexpressed in some human multiple myelomas due to its chromosomal translocation to the immunoglobulin locus (4). Up-regulation of c-maf expression was also found in mouse melanoma cells that were induced by the insertion of the melanoma-associated retrovirus (5).

The Maf protein bears a basic leucine zipper (bZip) structure at its carboxyl terminus, through which the protein forms homodimers (1). Maf preferably binds to the palindromic DNA sequences TGCTGACTCAGCA and TGCTGACGTCAGCA, which we have denoted as Maf recognition elements (MAREs) (6–8). The MAREs are related in sequence to the phorbol 12-O-tetradecanolate-13-acetate-responsive element (TRE; TGACTCA) and to the cyclic AMP-responsive element (TGACGTCA), to which the AP-1 and CREB/ATF family of bZip transcription factors bind. The DNA binding specificity of the Maf homodimer thus overlaps partly with that of the AP-1 transcription factor (6, 9). AP-1 is composed of homo- and heterodimers of the Jun and Fos family of proto-oncogene products. Maf also can form heterodimers with both Jun and Fos. The Maf/Jun and Maf/Fos heterodimers preferably recognize nonpalindromic DNA sequences composed of each half-site of MARE and TRE.

It has been shown that MARE, TRE, and the related cis-DNA elements exhibit their unique and important roles in transcriptional regulation through their interaction with homo- and heterodimers of bZip-bearing transcription factors. For example, the antioxidant-responsive element in the glutathione S-transferase P gene promoter, which contains TRE and is very similar to the consensus MARE sequence, has been shown to be regulated by both Maf and AP-1 factors (10). Such regulation may participate in the transcription of glutathione S-transferase P in response to a wide variety of extracellular stimuli. In contrast, the MARE found in the interleukin-4 gene promoter is quite different from TRE and is bound by Maf but not by Jun or Fos (11), which confers a lineage-specific expression of interleukin-4 in the Th2 subset of helper T-cells.

The bZip domains of v-/c-Maf are indispensable in their DNA binding and transactivation abilities. They are also highly conserved throughout evolution among the Maf family members MafA/L-Maf, MaB, and Nrl (3, 6). The amino-terminal domain of Maf is also conserved among these family members and is rich in acidic and serine, threonine, and proline residues. The central part of v-/c-Maf contains a cluster of histidine residues
Transactivation and Transformation by Maf

and three clusters of glycine residues. The histidine cluster is also found in MafA/L-Maf and MafB, but the glycine clusters are unique to the v-c-Maf protein. Structure-function analysis has revealed that the deletion of the amino-terminal regions of Maf inhibits its transactivator function and transforming ability (3, 8), suggesting that these two biological activities may be correlated. This notion, however, is disputed by the artificial mutant Maf protein termed Q5H, which has a glutamine to histidine substitution in the hinge between the basic domain and the leucine zipper. Q5H is significantly better at transactivation despite the fact that its transactivation activity is similar to that of the wild type Maf protein (3, 8). Thus, the precise role of the transactivator function of Maf in cell transformation is unclear to date. The downstream target gene(s) of Maf (if any) that are responsible for cell transformation are also unknown.

To elucidate the mechanism by which Maf transforms cells, we constructed a series of chimeric molecules that contained portions of Maf fused with heterologous transactivator, transrepressor, or dimerization domains. These molecules were tested for their transactivation and transforming activities. We also attempted to determine whether the downstream target gene(s) of Maf are the same as those of Jun oncoproteins.

EXPERIMENTAL PROCEDURES

Plasmid Construction—To make pEF/Gal4-VP16, the HinII (SacII)-EcORI fragment containing the SV40 promoter and the rabbit β-globin intron sequence of the pSG-GalVP plasmid was replaced by the HindIII-EcoRI fragment of the human polypeptide chain elongation factor 1α promoter derived from the pEF-BOS plasmid (12). The pSG-GalVP plasmid is a pSG5 (Stratagene)-based eukaryotic expression vector that contains an EcoRI-BamHI fragment that links the DNA-binding domain (DBD) of Gal4 (amino acids 1–147) to the acidic transactivator domain of pVP16 (amino acids 413–490) by a HindIII-BglII linker, AAGGCTGACCT.

pEF/Gal4-Maf(CD4) was constructed as follows. First, a HindIII restriction site was introduced at the 5′-end of the open reading frame of c-maf by a polymerase chain reaction (PCR) using primers (5′-aggagttAGGCTAGCCGAAATCCTGGAACACCGGTCTC-3′ and 5′-ACGGAGCTGAGGGCGCCGTCATA-3′). The PCR fragment was digested with HindIII and Ncol and then digested into the HindIII-Ncol site of pCD4(3), which generates a pUC9-c-maf-Cd4, which encodes amino acids 1–254 of chicken c-Maf. The HindIII-BamHI (nucleotide 1036 of v-maf) fragment was then introduced into the pEF/Gal4-VP16 plasmid by replacing its HindIII-BamHI fragment.

pEF/Gal4-DBD was then made from pEF/Gal4-Maf(CD4) by deleting the HindIII-XhoI fragment by T4 DNA polymerase treatment and self-ligation. The Ncol restriction site is derived from a synthetic linker sequence that was inserted into the BstEII site at nucleotide 756 of v-maf.

The plasmids that contain maf Pt, which is a nearly full-length version of v-maf (pRAM-GEM), and the amino-terminally deleted maf mutant N55 (pNd5) have been described (3).

For the Mxi1-Maf fusion, human mxi1 cDNA (13) was amplified by reverse transcriptase-PCR from the total RNA of the TIG1 cell line using specific primers (5′-ttaggatTCGATCCCTGCAAGGCG-3′ and 5′-tagagttGCTATCATGAACCCAATTATAG-3′). The product was then digested with SphI and subcloned into the SphI site of the pUC118 vector in the HindIII-EcoRI direction. An MluI linker was inserted into the HindIII site of the polylinker. The BglII site of the mxi1 cDNA and the Ncol site (nucleotide 53 of v-maf) of pRAM-GEM plasmid were then ligated after T4 polymerase treatment. An MluI fragment coding for a fusion protein consisting of amino acids 1–70 of Mxi1 that contains the Sin3 interaction domain (SID) fused to amino acids 19–369 of v-Maf was excised and inserted into the pGEM-4/MluI linker (vector (14) to make pGEM-4/Mlu/mxi1-maf.

For the VP16-N55 chimera, a DNA fragment containing the VP16 transactivator domain was amplified from the pSG-GalVP plasmid by PCR using specific primers (5′-aggagttGCTATCATGAACCCAATTATAG-3′ and 5′-ggagttGCTATCATGAACCCAATTATAG-3′). It was then digested with Ncol and Sall. A SalI restriction site was introduced at the 5′-end of the open reading frame of pN55 by PCR using a specific primer (5′-aggagttGCTATCATGAACCCAATTATAG-3′ and 5′-ggagttGCTATCATGAACCCAATTATAG-3′). The resulting fragment was then inserted into the pRAM-GEM plasmid by replacing its Ncol-MluI fragment to make pGEM-4/Mlu1GNC4.

For domain exchange between v-Maf, v-Jun, and GNC4, an Xhol restriction site was generated at the first leucine residue of the zipper structure by site-directed mutagenesis (16) using oligonucleotides (5′-GCTTGAGTCGCAATGGATCC-3′ for v-Maf, 5′-GATCTTTCCTCGAGCCTGACATC-3′ for v-Jun, 5′-CAAGCATGTCGACATTACCGATAG-3′ for GNC4). The plasmid carrying the v-jun coding region has been described previously (6). The maf-GNC4 chimera (pGEM-4/Mlu1maf-GNC4) was generated from the v-maf (+XhoI) and GNC4(+Xhol) constructs. The N65-GNC4 chimera (pGEM-4/Mfu1maf-GNC4) was constructed from pNd5 and pGEM-4/Mlu1maf-GNC4. A plasmid coding for a fusion protein containing the GNC4 zipper region and the amino-terminally deleted version of v-Jun (pGEM-4/Mfu1ndjun-GNC4) was constructed from pGEM-4/Mfu1GNC4(+Xhol) and pGEM-4/Mfu1ndjun(+Xhol). The latter construct was generated by PCR using a specific primer (5′-ggagttGCTATCATGAACCCAATTATAG-3′ and 5′-CAAGCATGTCGACATTACCGATAG-3′) and the SP6 sequencing primer followed by NoI and EcoRI digestion and subcloning into the NoI-EcoRI site of pRAM-GEM.

Constructions of point mutants of the maf-v gene were previously described (3).

Gel Mobility Shift Analysis—The deletion and fusion constructs described above were linearized by digestion with the appropriate restriction enzyme and then transfected and translated in vitro using the TNT Coupled Wheat Germ Extract System (Promega). The resulting extracts were subjected to gel mobility shift analysis as described previously (6). A double-stranded competitor oligonucleotide spanning the aCE2 element of chicken α-crystallin promoter (17) was made from 5′-GATCCAGGTATCGCTGCAAGACCCACGTGTCAGATATAAAGGACGTGTCAGTAAAGGACGCTGTCAGCAGATAGCCAG-3′ and 5′-GTCGATCTGAGGCCGTAATAGACAGCAGCTGTCAGTAAAGGACGCTGTCAGCAGATAGCCAG-3′. These oligonucleotides were used as probes or competitors and have been described previously (6, 18).

Luciferase Assay—For eukaryotic expression, each MluI fragment containing one of the deletion or fusion constructs described above was inserted into the unique BstEII site of the pEF-BstEII expression vector (19), which is a derivative of pEF-BOS. The pG4x5STATA/luc plasmid that contains five copies of the Gal4-binding site was a gift from K. Igarashi (Osaka University, Osaka, Japan) (20).

The construct 3xMARE/RBGP-luc that contains three copies of MARE (oligonucleotide 7) and the rabbit β-globin minimal promoter has been described (8, 19). Three copies of a double-stranded oligonucleotide containing an E-box (5′-GATCCCCCATGACGCTGTCAGTGAATAGACAGCAGCTGTCAGTAAAGGACGCTGTCAGCAGATAGCCAG-3′ and 5′-GTCGATCTGAGGCCGTAATAGACAGCAGCTGTCAGTAAAGGACGCTGTCAGCAGATAGCCAG-3′) were introduced into the 5′-end of the MARE-containing oligonucleotides of 3xMARE/RBGP-luc to make 3xE-box/3xMARE/RBGP-luc. pEF-RLuc, used to normalize transfection efficiencies, has been described previously (18).

CEF cells (1.2 × 10^6) grown on 60-mm dishes were transfected with 2.5 µg of plasmid DNA (0.2 µg of reporter plasmid, 2.0 µg of expression plasmid, and 0.3 µg of pEF-RLuc plasmid) using 6.25 µL of Superfect Transfection Reagent (Qiagen) as recommended by the manufacturer. The cells were harvested 18 h post-transfection, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

To monitor expression of Gal4 fusions and mutant Maf proteins, aliquots of total extracts of the transfected CEF cells were analyzed by Western blotting using one of the following antibodies (sc-510; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-v-Maf serum (3), or anti-c-Maf serum (sc-7886; Santa Cruz Biotechnology).

Focus Formation Assay—For the focus formation assay, Mlu1 fragments containing chimeric Maf constructs were introduced into the unique Maf site of the replication-competent avian retroviral vector pBR2 (3) or its modified version, pBR9 (18). The NorI cassette plasmid was activated by the retroviral vector}

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Transactivation and Transformation by Maf

**RESULTS**

The Amino-terminal Domain of Maf Bears the Transactivator Function—The amino-terminal two-thirds of v/c-Maf contain the acidic and serine/threonine/proline-rich region and clusters of histidine and glycine residues (Fig. 1A). We previously reported that deleting all of these parts of Maf resulted in a great reduction of its transactivating activity, although its ability to bind to MARE was impaired (6, 8). To define the transactivation potential of the amino-terminal region of Maf, we generated a plasmid that encoded a fusion protein consisting of the first 254 amino acids of c-Maf linked to the DBD of the Gal4 yeast transcription factor (Fig. 1A). As a control, a fusion construct consisting of the DBD of Gal4 linked to the strong transactivator domain of the VP16 is comparable with full-length Maf (3). ND5 lacks two-thirds of the amino-terminal portion and has been shown to be unable to transform CEF cells (3). To test if the transactivator domain of Maf can be exchanged with heterologous transactivator domains, the VP16 activator domain was fused to the bZip domain contained in ND5 to create VP16-ND5. Another chimeric construct, Mxi1-Maf, fuses the SID of the Mxi1 transcriptional repressor protein to the PT form of Maf. SID has been shown to interact with Sin3 proteins to recruit the histone deacetylase complex and to repress transcription mediated by several types of transactivation domains (22, 23). These four constructs were each transcribed and translated in vitro and tested for binding to the MARE sequence by gel mobility shift analysis. As shown in Fig. 2B, all proteins bound efficiently to the MARE-containing oligonucleotide probe. The translation products of the Mxi1-Maf construct also resulted in a DNA-protein complex formed by Mxi1-Maf fusion protein is indicated by an asterisk.

**Transcriptional Activation Is Required for Maf-induced Cell Transformation**—We next tested the relationship between the abilities of Maf to activate transcription and to transform CEF cells. For this purpose, we designed a series of mutant Maf constructs that are shown schematically in Fig. 2A. MafPT is a nearly full-length version of Maf whose transforming ability is comparable with that of full-length Maf (3). ND5 lacks two-thirds of the amino-terminal portion and has been shown to be unable to transform CEF cells (3). To test if the transactivator domain of Maf can be exchanged with heterologous transactivator domains, the VP16 activator domain was fused to the bZip domain contained in ND5 to create VP16-ND5. Another chimeric construct, Mxi1-Maf, fuses the SID of the Mxi1 transcriptional repressor protein to the PT form of Maf. SID has been shown to interact with Sin3 proteins to recruit the histone deacetylase complex and to repress transcription mediated by several types of transactivation domains (22, 23). These four constructs were each transcribed and translated in vitro and tested for binding to the MARE sequence by gel mobility shift analysis. As shown in Fig. 2B, all proteins bound efficiently to the MARE-containing oligonucleotide probe. The translation products of the Mxi1-Maf construct also resulted in a DNA-protein complex of lower mobility. The identity of this complex is unknown at present, but it could be the Mxi1-Maf fusion protein bound to the endogenous histone deacetylase complex present in the translation system we used.

We next tested the ability of these four constructs to regulate transcription. Each construct was introduced into a eukaryotic expression vector and co-transfected into CEF cells together with the luciferase reporter plasmid that drives transcription under the control of the rabbit β-globin promoter (RBGP) TATA-box and three tandem MARE repeats (3xMARE/RBGP-
Luciferase activities were then measured. We found that all Maf constructs were efficiently translated in the transfected cells as revealed by Western blot analysis using anti-v-Maf serum (Fig. 3). We also found that they all accumulated in the nucleus of the transfected cells as observed by immunofluorescent staining using anti-v-Maf serum (data not shown).

The luciferase data are summarized in Table I. Luciferase activity in cells co-transfected with 3xMARE/RGBP-luc and the empty expression vector was set at 1. The luciferase activity was increased by about 20-fold by the co-expression of Maf(PT) protein, while deletion of the amino-terminal two-thirds of Maf (ND5) resulted in complete loss of transactivation as previously observed using NIH3T3 mouse fibroblast cells (8). As expected, the VP16-ND5 chimera activated transcription more efficiently (160-fold) than Maf(PT), which is consistent with the transactivation potentials of Gal4-Maf(CD4) and Gal4-VP16 seen previously (Fig. 1B). On the other hand, Mxi1-Maf was transcriptionally inert, indicating that the presence of Mxi1-SID successfully blocks the function of the Maf transactivation domain. However, it was not clear whether Mxi1-Maf acts as a transcriptional repressor, because the basal activity of the 3xMARE/RGBP-luc reporter plasmid is relatively low in CEF cells. Thus, in order to test the repressor function of Mxi1-Maf, we used another reporter plasmid, namely 3xE-box/3xMARE/RGBP-luc. This reporter plasmid contains three copies of the E-box sequence and has a 7-fold higher basal activity when transfected into CEF cells compared with 3xMARE/RGBP-luc (Table I). Consistent with the patterns seen previously with the 3xMARE/RGBP-luc reporter, the luciferase activity of 3xE-box/3xMARE/RGBP-luc was enhanced by co-transfection with either Maf(PT) (5-fold) or VP16-ND5 (56-fold). In addition, however, co-transfection of Mxi1-Maf resulted in a significant reduction of luciferase activity, indicating that it does act as transcriptional repressor.

We then tested the abilities of these Maf constructs to transform CEF cells by introducing them into a replication competent avian retroviral vector that was then transfected into CEF cells. The recombinant viruses were then assayed for their focus-forming activities. As previously demonstrated, the recombinant virus carrying Maf(PT) could induce foci on CEF cells, while the ND5 deletion mutant could not (Fig. 4 and Table I) (3). The VP16-ND5 containing virus, however, induced foci more efficiently and rapidly (5–20 × 10^3 focus forming units/ml, 10–14 days after infection) than the Maf(PT) virus (2–3 × 10^3 focus forming units/ml, 13–18 days after infection). Thus, the amino-terminal domain of Maf can be replaced by the heterologous transactivator domain of VP16, and the magnitude of the transforming ability seems to correlate with the transactivation potential. In contrast, the Mxi1-Maf-containing virus could not induce foci despite the fact that it contained the full Maf sequence. The most likely explanation for this observation is that the presence of the dominant repressor domain resulted in the inability of Mxi1-Maf to transactivate, which in turn abrogated its potential to transform CEF cells. Thus, in terms of the role of Maf in cell transformation, the Maf amino-terminal region acts only as a transactivator domain, and other additional functions, if any, are dispensable. These results together also strongly suggest that transactivation through MARE is necessary for transformation by the Maf oncoprotein.

**Heterodimer Formation Is Not Necessary for Maf-induced Cell Transformation**—We and others have previously shown that Maf can associate with other bZip transcription factors such as Jun, Fos (6, 7), and Bach,^2^ and that this results in the formation of heterodimers with altered DNA binding specificities. However, it is unclear whether heterodimer formation with such bZip factors is necessary for Maf’s ability to transform cells. To address this question, the leucine zipper region of Maf, which should define Maf’s dimer forming specificity, was replaced with that from the GCN4 yeast protein (Fig. 5A). This chimeric molecule, Maf-GCN4, is expected to form dimer with

| Transactivation | Focus formation |
|-----------------|-----------------|
| 3xMARE/RGBP-luc | 3xE-box/3xMARE/RGBP-luc |
| Vector          | 1               | 7.1 (1)           | –               |
| Maf(PT)         | 20.9            | 37.6 (×5.3)       | +               |
| ND5             | 1.7             | ND                | –               |
| VP16-ND5        | 159.0           | 400.4 (×56.4)     | ++              |
| Mxi1-Maf        | 0.6             | 1.0 (×0.16)       | –               |

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^2 M. Nishizawa, unpublished observations.
The indicated proteins were translated in vitro for the Maf-GCN4 chimera.

B

serum. Accumulation of Maf(PT) and Maf-GCN4 was detected by Western blot using anti-c-Maf antibodies that specifically recognized Maf(PT) or Maf-GCN4 when their expression plasmids were transfected into CEF cells (Fig. 5B). Accumulation of Maf(PT) and Maf-GCN4 in transfected CEF cells revealed by Western blot using anti-c-Maf serum indicates that Maf(PT) and Maf-GCN4 remain intact after transfection and translate into protein. Indeed, as expected, it could efficiently bind to MARE as homodimers when it had been transcribed and translated in vitro (Fig. 5B). It activated the transcription of the MARE-containing reporter plasmid containing multiple oligonucleotide 7, which is efficiently bound by Maf homodimer but not by Jun homodimer (see Fig. 7A). As expected, the DNA-binding preferences of these chimeric bZip molecules by using a set of oligonucleotides that contained AP-1/MARE-related sequences as competitors (Fig. 7C). As expected, the DNA-binding preferences of ND5-GCN4 and NDJun-GCN4 were very similar to that of Maf and Jun, respectively.

We also tested whether ND5-GCN4 and NDJun-GCN4 chimera can specifically repress transactivation by Maf and Jun, respectively. Luciferase activity of CEF cells transfected with a reporter plasmid containing multiple oligonucleotide 7, which is efficiently bound by Maf homodimer but not by Jun homodimer (see Fig. 7C), was increased by co-expression of Maf but not by Jun (Fig. 8). The Maf-induced luciferase activity was significantly decreased by co-transfection of expression plasmid for ND5-GCN4 but not for NDJun-GCN4, demonstrating specific repression of Maf’s transactivation by ND5-GCN4. Unfortunately, our attempt to demonstrate repression of Jun activity by NDJun-GCN4 was not successful, because endogenous transcriptional activity using reporter plasmid containing AP-1-like elements (oligonucleotide 11 or 23; see Fig. 7C) was too high in CEF cells to detect transactivation by Jun.

We next tested the effect of co-expressing these chimeric molecules on the abilities of Maf and Jun to transform. In order...
to express two exogenous genes in a cell, we developed a replication-competent avian retrovirus vector system that contains an IRES (18). After transfection into CEF cells, we expect one gene to be expressed from fully spliced subgenomic RNA and the other from any of the bicistronic RNA species by utilizing the IRES. We constructed a set of retroviral expression plasmids, transfected them into CEF cells, and assayed for focus formation. As shown in Fig. 9 (bottom), introduction of the v-myc nuclear oncogene into CEF cells resulted in transformed foci. Co-expression of either ND5-GCN4 or NDJun-GCN4 utilizing IRES had no effect on the efficiency of cell transformation by v-Myc, indicating that these chimeras were not toxic to cell growth. In contrast to the case of Myc, co-expression of ND5-GCN4 together with Maf resulted in a great reduction of focus formation by Maf (Fig. 9, top). NDJun-GCN4 also inhibited the transformation by Jun (Fig. 9, middle). The reason for this apparent inconsistency will be discussed below.

Correlation of Deficiencies in Cell Transformation and Specific DNA Binding of Point Mutants of Maf—In our previous study, we constructed a series of amino acid substitution mutants of Maf protein within its DNA-binding domain (Fig. 10A) and have shown that they lost transforming abilities except for the Q5H mutant (3). The most likely explanation for their inability of cell transformation is their loss of DNA binding activity. To confirm this, we produced these mutants using an in vitro translation system (Fig. 10B) and subjected them to gel mobility analysis (Fig. 10C). Expectedly, R22E and K19E mutants, which have amino acid substitutions at the conserved basic residues (Arg or Lys) in the DNA binding domain with acidic amino acids (Glu), completely lost affinities to any of the oligonucleotide probes used (probes 11, 1, and 7). The other transformation-defective mutants, A14D, A14V, and R10D, had only marginal affinities to the probe 11. However, A14D and A14V mutants retained affinity to probe 1 and, surprisingly, had higher affinity to the probe 7 than wild type Maf. R10D mutant also had higher affinity to probe 1. Therefore, amino acid substitutions in the DNA-binding domain of these three mutants resulted in changes of their DNA binding specificities. Supposing that DNA-binding activity is a prerequisite for transforming ability of Maf, these results indicate that...
Transactivation and Transformation by Maf

In this study, we first definitively established that the amino-terminal two-thirds of Maf acts as a transactivator domain. This is consistent with our previous observation that deletion of this domain causes Maf to lose its transactivation activity and transforming ability (3, 8). The acidic and serine/threonine/proline-rich region in this domain is well conserved throughout evolution among the Maf family members, which include v-/c-Maf, MafB, MafA/L-Maf, and Nrl (1, 19, 28-31). The central part of v-/c-Maf is less conserved, but in chickens, mice, rats, and humans it contains clusters of glycine and histidine residues (2, 4, 32, 33). The histidine cluster is also found in MafB and MafA/L-Maf. This conservation of amino acid sequences in the amino-terminal domains of Maf family members suggests that this region is important not only as a transactivator domain but that it may also have other unknown functions. We have shown here, however, that the ability of Maf to transactivate and to transform can still occur efficiently when the amino-terminal domain of Maf has been exchanged with the heterologous transactivator domain of VP16. This indicates that the amino-terminal domain of Maf is only important for cell transformation because of its ability to serve as a transactivator domain. This notion is supported by the observation that attaching SID, which masks transactivator function, to the full-length Maf protein results in the complete inability of Maf to transform. These observations do not negate the possibility that the amino-terminal portion of Maf may play other roles in the physiological function of Maf. For example, it may serve as an interaction surface with other cellular factors. Nevertheless, our observations clearly demonstrate that transactivation through MARE is necessary for Maf-induced cell transformation.

The sequence conservation of the Maf family proteins indicates that they are all transactivators (19, 30, 31, 34). Our comparative study has previously demonstrated that MafB is a weaker transactivator than v-/c-Maf and is also less potent in inducing cell transformation (19). In this study, we found that the VP16-ND5 chimera, which consists of the C-terminal part of Maf fused to the VP16 transactivation domain, is better than Maf in its ability to transactivate and transform. These observations together suggest that the magnitude of a given Maf protein’s transactivating potential via MARE might correlate with its transforming ability. Disputing this notion, however, is the artificial mutant Maf protein termed Q5H, which has a glutamine to histidine substitution in the hinge between the basic domain and the leucine zipper. Q5H is much better in transforming cells than wild type Maf despite the fact that it has a similar transactivation activity (3, 8). We also found that MafA/L-Maf is a more potent transformer than v-/c-Maf despite the fact that it was less active as a transactivator.3 Thus, although transactivation through MARE is necessary for transformation, it seems that other unknown function(s) of Maf may shape its transforming potential.

In many cases, bZip transcription factors selectively form heterodimers with other bZip factors to regulate transcription. As far as we know, Maf forms heterodimers with Jun, Fos (6, 7), Bach,2 and some members of the ATF/CREB family of proteins.2 Upon heterodimerization, the DNA binding specificity of the dimer changes, and thus it should regulate a different set of target genes. In the present study, we showed that the Maf-GCN4 chimera, whose leucine zipper domain has been replaced by that of the GCN4 yeast protein, could efficiently induce cell transformation. Thus, heterodimerization with other bZip proteins is not necessary for Maf’s ability to transform. This is consistent with our previous observation that we could not detect any proteins associated with Maf when Maf-transformed CEF cells were immunoprecipitated with anti-Maf antiserum (2).

These results strongly suggest that the Maf homodimer targets specific gene(s) that are necessary in the induction of transformation. In the first step taken to identify these gene(s), we wanted to know whether the target gene(s) of Maf are shared by Jun. This question arose because the DNA binding specificities of Maf and Jun overlap with but are distinct from each other, as we have previously shown (6). For this purpose, we examined the ability of ND5-GCN4 and NDJun-GCN4 to suppress transformation. These two chimeras were designed to interfere only with transcription of the genes targeted by homodimers of Maf and Jun, respectively. One of the important

3 M. Nishizawa and K. Kataoka, unpublished observations.
observations made with this experiment is that these two constructs could not repress the v-Myc-induced transformation. Previous reports have also indicated that dominant negative mutants of Jun or Fos are also not able to interfere with the transforming ability of Myc (35). These observations together suggest that transcriptional activation through either MARE or AP-1 is not necessary for cell transformation by the Myc oncoprotein.

As we expected, ND5-GCN4 and NDJun-GCN4 successfully repressed transformation by Maf and Jun, respectively. However, we also found that the co-expression of NDJun-GCN4 resulted in a great reduction in Maf’s focus forming efficiency while, in contrast, ND5-GCN4 had no effect on transformation by Jun. One possible explanation of this is that Maf and Jun share common target gene(s) regulated by an AP-1/MARE-like cis-element that has a higher affinity for Jun than for Maf. Thus, NDJun-GCN4 can compete for Maf in binding to the element, but ND5-GCN4 cannot efficiently compete with Jun. In any case, the fact that NDJun-GCN4 can suppress Maf-induced transformation clearly indicates the existence of common target gene(s) of Maf and Jun. This idea is also supported by our finding that some of transformation-defective point mutants of Maf lost binding affinity to the DNA sequence that is also bound by Jun, in spite of the fact that they retained binding affinity to Maf-specific DNA elements. These mutant Maf proteins probably cannot no longer activate such essential common target gene(s). One such candidate target is the hepatic-binding epidermal growth factor-like growth factor, which is a common target gene(s) regulated by an AP-1/MARE-like element, but ND5-GCN4 cannot efficiently compete with Jun. One possible explanation of this is that Maf and Jun share common target gene(s) of Maf and Jun. This idea is also supported by our finding that some of transformation-defective point mutants of Maf lost binding affinity to the DNA sequence that is also bound by Jun, in spite of the fact that they retained binding affinity to Maf-specific DNA elements. These mutant Maf proteins probably cannot no longer activate such essential common target gene(s). One such candidate target is the hepatic-binding epidermal growth factor-like growth factor, which has recently been reported to be induced by both Jun and Maf and to be oncogenic in itself (36).

Other target genes that are specific to Maf should also exist, because the CEF cells transformed by Maf differ morphologically from those transformed by Jun. Furthermore, Maf induces musculoaponeurotic fibrosarcomas in chickens (2), whereas Jun induces sarcomas (37). Although some physiology of target genes of Maf, such as interleukin-4 in the TH2 subtype of helper T-cell (11) and the crystallin genes in lens fiber cells (24, 25), have been identified, the target genes responsible for such transformed phenotypes have as yet to be identified. To fully understand the mechanism by which Maf transforms cells, the identification and analysis of target genes is necessary. The Maf fusion constructs containing either the VP16 transactivator domain or the SID repressor domain that we used in this study will be powerful tools in the isolation of such target genes. Combining the VP16 and SID fusions together with an inducible expression system may help greatly in selecting and identifying downstream target genes based on their expression levels. Once identified, the responsiveness of such genes to Maf and Jun may serve as criteria that allow us to define them either as effector genes for cell transformation itself or as modulator genes for the transformed phenotype.

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