Oncogenic Point Mutations Induce Altered Conformation, Redox Sensitivity, and DNA Binding in the Minimal DNA Binding Domain of Avian Myeloblastosis Virus v-Myb

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The expression of the c-myb proto-oncogene is restricted to a limited range of differentiating cell types (for recent reviews, see Refs. 1 and 2). Myb's function is particularly important in early stages of hematopoiesis, where it plays a crucial but poorly defined role in maintaining the proliferative state of immature cells (reviewed in Refs. 3 and 4). High levels are expressed in immature cells of all lineages, and the expression is strongly down-regulated during terminal differentiation. Aberrant overexpression of c-Myb inhibits differentiation of hematopoietic precursor cells (reviewed in Ref. 5), while antisense oligonucleotides directed at c-myb inhibit their proliferation (6–8). Mice homozygous for a c-myb disruption die from multiple hematopoietic defects during embryonic development (9), and transgenic mice with T cell-specific expression of a dominant interfering allele of Myb display partially blocked thymopoiesis and diminished proliferation of mature T cells (10). The molecular mechanisms producing these phenotypes are poorly understood, in particular when it comes to Myb-regulated target genes with a role in proliferation and differentiation. Candidate target genes have recently been reviewed (2).

The c-myb gene encodes a 75-kDa sequence-specific DNA binding transcription factor with at least three functional domains (2). The DNA binding domain (DBD) located near the amino terminus is a highly conserved tryptophan-rich region composed of three imperfect repeats (R1, R2, and R3), each related to the helix-turn-helix motif (11–13). Each repeat appears to have a distinct function. R3 is a fully folded domain mainly responsible for the sequence-specific recognition of the AAC core in the binding site (13, 14). R2 is more flexible and seems to undergo a conformational change upon binding to DNA, possibly to allow the protein to adapt to a range of flanking sequences (15–17) (see also Carr et al. (18)). R1 also contains a highly oxidizable cysteine implicated in redox control (16). An NMR-derived structure of a mouse R3R3-DNA complex was recently reported (14). The role attributed to R1 has been a stabilization of the complex through electrostatic interactions (19–21). An acidic transactivation domain is found centrally located in the protein, and a large carboxyl-terminal region appears to have a negative effect on Myb's transactivation and DNA binding functions. A recent report suggested that several subdomains may cooperate to form a functional transactivation domain, implying that the precise borders of this domain remain to be elucidated (22).

Distinct mechanisms of oncogenic activation seem to operate in the two isolated avian v-myb-containing retroviruses E26 and avian myeloblastosis virus (AMV) (reviewed in Refs. 1, 4, and 5). Both contain truncated v-myb oncogenes. E26 encodes an amino- and carboxyl-terminally truncated Myb protein that is fused to the v-Ets oncoprotein, while AMV encodes a truncated Myb protein that displays a number of specific amino acid substitutions relative to c-Myb. E26 is able to transform multipotent hematopoietic cells and causes erythroblastosis and a low level of concomitant myeloblastosis in chickens, while AMV

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MATERIALS AND METHODS

Expression and Purification of Myb Proteins—The minimal DNA binding domain of the chicken c-Myb protein, R1R2, and two mutant derivatives were expressed in E. coli BL21(DE3)pLyS8 strain, and proteins were purified as described previously (12).

Mutagenesis—Site-directed in vitro mutagenesis was performed as described elsewhere (12, 27). Plasmids from positive clones, identified by the electrophoretic mobility shift assay (28), with the modifications described in elsewhere (12). The basic duplex oligonucleotide “MRE primer” (5'-GGGCCCTAA-3') was used for polynucleotide kinase and [γ-32P]ATP. After annealing and fill-in, all probes got identical specific activities and therefore directly comparable intensities in electrophoretic mobility shift assay. Labeled duplex oligos were purified by polyacrylamide gel electrophoresis.

Electrophoretic Mobility Shift Assay—DNA binding was monitored by electrophoretic mobility shift assay (38). The MRE consensus sequence was used (Scheme 1) (30). Labeled oligonucleotides were obtained by end-labeling of a small common

SCHEME 1

- MRE (mim) 5'-GGCTATATACGCGTTTCTAGGC-3'
- 3'-CGTAATACGCTGAAACCTCGCA-5'

GG:
- 5'-GGCTATATACGCGTTTCTAGGC-3'
TG:
- 5'-GGCTATATACGCGTTTCTAGGC-3'
GT:
- 5'-GGCTATATACGCGTTTCTAGGC-3'
TT:
- 5'-GGCTATATACGCGTTTCTAGGC-3'

Table 1

Base numbering in MRE 123456789

RESULTS

The AMV-specific Mutations in R2 Alter the Conformation of the DNA Binding Domain—To assess whether the three AMV-specific point mutations in R2 had any effect on the structure of the minimal DNA binding domain of Myb (R1R2), we first studied purified DBDs by fluorescence spectroscopy taking advantage of the high content of tryptophans in these domains conferring intrinsic fluorescent properties to the proteins. Three subdomains were expressed in E. coli and purified, the chicken wild type R2R3 protein (designated R2R3[wt]) and two mutated R2R3[AMVNHD] harbored all three AMV specific mutations in R2 (R1R2) and V117D, while R2R3[AMVNHD] harbored only the two latter (L106H and V117D). Fig. 1 shows the locations of the introduced mutations relative to secondary structure elements of

SCHEME 2

- NT: 5'-GCATTATAACGCCTTCTTTCTAGGC-3'
- GT: 5'-GCATTATAACGCCTTCTTTCTAGGC-3'
- TT: 5'-GCATTATAACGCCTTCTTTCTAGGC-3'
that the DNA-induced conformational change in R2R3[AMV]
a more compact structure than its wild type homologue, and
suggested that the AMV-like DNA binding domain might have
previously (16), the emission maximum of R2R3[wt] was shifted
denatured protein for normalization (Fig. 2). As described pre-
denatured, native, or DNA-bound form, using the spectrum of
of the two subdomains in three states, guanidinium chloride-
were closer to the spectrum of the DNA-bound
state than the emission spectrum of native R2R3[wt]. This
could be more modest than in R2R3[wt].
To obtain more direct evidence for this hypothesis, we per-
formed fluorescence quenching experiments to monitor if the
AMV-specific mutations induced any alteration in the average
exposure of tryptophans in the R2R3-protein. As seen from the
Stern-Volmer plot in Fig. 3, both R2R3[AMV-NHD] and
were quenched significantly less by the neutral
quencher acrylamide than R2R3[wt], supporting our hypothesis
of a more compact structure in the mutants relative to the wild
type protein. After binding to DNA, all three forms showed a
similar tryptophan exposure, indicating more similar confor-
mations in the DNA-bound state. Since a purified R3 domain
was found to be less quenched than R2R3[wt] (results not
shown), the tryptophans in R3 must be less exposed to solvent
than the average of all six tryptophans in R2R3[wt]. Hence,
the three tryptophans in wild type R2 must be significantly
more exposed to solvent than their R3 homologues in order for R2
to make a major contribution to the increased slope of the Stern-
Volmer plot of R2R3[wt]. This exposure is then reduced upon
some critical mutations in R2, both the AMV-specific mutations
studied here and the previously reported C130V mutation (des-
noted C43V in Myrset et al. (16)). The L106H and V117D
mutations made the major contributions to this conformational
effect, since no significant differences were detected between
the two proteins that differed with respect to the I91N muta-
tion. For this reason, in some experiments below only R2R3[wt]
and R2R3[AMV-HD] are compared.
If the difference in conformation between R2R3[AMV-HD] and
R2R3[wt] were sufficiently large, the two proteins might be
expected to display differences in their proteolytic sensitivity.
The two purified proteins were therefore subjected to limited
proteolysis by chymotrypsin. This protease was chosen since
none of the amino acid replacements in R2R3[AMV-HD] should
affect the specificity of the enzyme. The band pattern of pro-
teolytic products revealed distinct differences between
R2R3[AMV-HD] and R2R3[wt] as indicated by arrows in Fig. 4.

**FIG. 1. Sequence and structure of the minimal DNA binding
domain of c-Myb. Panel A, the amino acid sequences of the chicken
Myb R2 and R3 repeats are shown in single-letter code and are aligned
by the three tryptophans in each repeat. The location of secondary
structure elements in the free protein determined by NMR (15) are
indicated by shaded boxes (α-helical regions) or by waves (disordered
flexible region). The AMV-specific mutations introduced in R2 (I91N,
L106H, and V117D) are shown above the wild type sequence.
Panel B, the three-dimensional structure of mouse R2R3 in complex with DNA
(structure elements in the free protein determined by NMR (15) are
indicated by shaded boxes (α-helical regions) or by waves (disordered
flexible region). The AMV-specific mutations introduced in R2 (I91N,
L106H, and V117D) are shown above the wild type sequence.

**FIG. 2. Emission spectra of Myb R2R3[wt] and R2R3[AMV] pro-
teins in denatured, native, and DNA-bound form. The emission of
2-μs protein denatured in 6 M guanidinium chloride, native in TEβ,
in TEβ complexed with 2 μM DNA (MRE(mim)), was monitored from
310 to 400 nm as described under “Materials and Methods.” The emis-
sion spectra of the Myb R2R3[wt], R2R3[AMV-NHD], and R2R3[AMV-HD]
proteins are indicated.
Both the total number and the positions of specific bands differed. This result strongly suggests that the AMV-specific point mutations in R2 indeed have a specific effect on the conformation of the DNA binding domain.

We also asked whether this conformational effect was specific to the AMV mutations or whether the conformation of the wild type protein was particularly sensitive to mutations in R2, such that any mutations in this domain would have a high probability of changing the conformation of R2. We analyzed a total of 15 different point mutations distributed over the entire R2 domain by running emission spectra on purified recombinant proteins complexed with 2 μM DNA were quenched by increasing concentrations of acrylamide. The results are presented as the ratio between unquenched and actual fluorescence (F0/F) as a function of acrylamide concentration, as described under “Materials and Methods.”

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previous findings that the same consensus recognition sequence holds for both c-Myb and AMV v-Myb (25). However, a clear difference was observed when the stability of the three protein-DNA complexes were compared. Under the specific conditions used to compare the decays of the complexes, the R2R3[wt]-DNA complex dissociated slowly and a substantial fraction remained after 20 min of competition with excess unlabeled DNA. In contrast, both the R2R3[AMV]-DNA complexes dissociated much more rapidly. After 5 min of competition less remained of the two R2R3[AMV]-DNA complexes than of the R2R3[wt]-DNA complex after 20 min (Fig. 5B). These data indicated that the conformational change induced by the AMV-specific mutations in R2 leads to a considerably reduced stability of the protein DNA complex.

To determine the specific to nonspecific binding constant ratios ($K_a/K_d$) (28), specific DNA complexes were titrated with increasing amounts of poly(dI-dC). As seen in Fig. 5C, the R2R3[AMVHD]-DNA complex was titrated at lower concentrations of poly(dI-dC) than the wild type protein. From the data shown a $K_a/K_d$ ratio of $6 \times 10^3$ was calculated for the R2R3[AMVHD] protein compared to $6 \times 10^4$ for the wild type protein. Assuming that the nonspecific DNA affinity is not affected, the AMV-specific mutations in R2 causes a 10-fold reduction in the specific DNA binding constant.

The AMV-specific Mutations in R2 Alter the Relative Stability of Complexes Formed with Subtypes of MREs—Previous in vitro studies of preferred binding sequences found no evidence for any alteration of sequence specificity as a result of the AMV-specific mutations (25). We have recently shown that strong MRE sequences with different configurations of guanine bases in the binding site for R2 gave remarkably different levels of transactivation in vivo at low expression levels of c-Myb (30). In vitro, corresponding differences between the binding sites variants could be seen only in analyses of protein-DNA complex stabilities. Since this phenomenon is related to the sequence of the half-site recognized by R2, we asked whether the AMV-specific point mutations in R2 had any influence on the relative complex stabilities when bound to different MRE variants.

Using three different MRE variants (TAACGG, TAAGCT, TAACGT) as binding probes, the stabilities of the complexes formed with R2R3[AMV] and R2R3[wt] were compared after competition with an excess of unlabeled MRE (Fig. 6). For all three probes the R2R3[wt] protein formed more stable complexes than R2R3[AMV]. A substantial fraction of all the R2R3[wt] complexes resisted 5 min of competition, but differences in stabilities allowed us to rank the complexes according to affinities in the order: TAACCG > TAAGCT > TAACGT (Fig. 6). This order parallels their ability to mediate transactivation in vivo (see below). In contrast, the R2R3[AMV] protein formed much less stable complexes. When bound to the TAACGG probe a visible fraction of the complexes resisted 5 min of competition (comparable to the R2R3[wt] protein in complex with the TAACGT probe). However, no visible complexes were seen even at the first time point (2 min of competition) when the R2R3[AMV] protein was bound to the TAACGT or to the TAACGT probes. Thus, single-G MRE variants form highly unstable complexes with R2R3[AMV], but are still reasonably stable when bound by R2R3[wt]. This suggests that the destabilization of the protein-DNA complexes caused by the AMV-specific mutations might be more severe with some MRE subclasses than with others.

The AMV-specific Mutations in R2 Alter the Redox Sensitivity of the DNA Binding Domain—The R2 repeat that is mutated in the AMV v-Myb also harbors a highly conserved redox-sensitive cysteine that was found to be essential for DNA binding, transformation, and transcriptional transactivation (36, 37).

The same cysteine is located in a disordered flexible region of R2 (15, 18). We have previously proposed that that this cysteine could function as a molecular sensor for a redox regulatory mechanism turning specific DNA binding on or off by controlling a DNA-induced conformational change in R2 (16). Having found that the AMV-specific mutations induced a more compact conformation in R2, we asked if this could influence the redox sensitivity of the conserved cysteine. First we titrated the two purified R2 proteins with increasing concentrations of the SH-specific oxidation reagent NEM. Specific DNA binding was abolished for all three proteins at sufficiently high NEM concentrations, but the titration showed a clear difference between the three variants with respect to NEM sensitivity since $R2R3[wt]$ was inactivated at significantly lower concentrations of NEM than both $R2R3[AMV]$ and $R2R3[AMVHD]$ (Fig. 7A). A corresponding difference was observed when the time course of inactivation was measured (results not shown). In both experiments a C130V mutant was unaffected, demonstrating the specificity of the alkylation.

We next examined Myb inactivation by treating the proteins with the SH-specific oxidation reagent diamide. As shown in Fig. 7B, specific DNA binding of R2R3[wt] was lost at lower concentrations of the SH reagent than observed for R2R3[AMV], suggesting a reduced redox reactivity as a result of the point mutations in R2. A time course experiment led to the same conclusion (results not shown). Finally, Myb inactivation by oxidation of the proteins with $H_2O_2$ was monitored. Again, specific DNA binding of R2R3[wt] was lost at lower concentrations than observed for R2R3[AMV] (Fig. 7C). Thus, we conclude that the conformational change induced by the AMV-specific point mutations in R2 changes the reactivity of the conserved cysteine in this repeat, making the DNA binding domain less susceptible to inactivation through modulation of the redox state of the critical cysteine.

The Effects of the AMV-specific Mutations in vivo Measured in a Yeast Effector-Reporter System—To assess the effects of altered DNA binding properties in a model in vitro situation, fusions between the DNA binding domains and the strong VP16 transactivation domain were expressed in yeast. The fusion gene was under control of the galactose-inducible GAL1–10 promoter in a centromeric low copy number plasmid, the latter to better mimic expression levels in a physiological in vivo situation. All reporters were high copy number plasmids (2 $\mu$m) containing the E. coli lacZ gene under control of the yeast
CYC1 minimal promoter. Insertion of three upstream MREs made each of them Myb-responsive. The reference reporter pBP19 with TAACGGAAC inserted has been described (34). Upon induction with galactose, the R2R3[wt]-VP16 and the R2R3[AMVNHD]-VP16 fusion proteins were expressed to the same level as judged by Western blot analysis of representative yeast extracts using a Myb-specific polyclonal antibody (data not shown). Since the only difference between the two fusion proteins was their DNA binding domains, the levels of induced β-galactosidase activity were taken as estimates of their in vivo DNA binding. Both fusion proteins were found to bind the pBP19 reporter plasmid and activate lacZ transcription. Induction of R2R3[wt]-VP16 resulted in 979 ± 65 β-galactosidase units, whereas only 395 ± 65 β-galactosidase units were found with R2R3[AMVNHD]-VP16 induced. These results suggest that the destabilization of the DNA binding caused by the AMV-specific mutations observed with the TAACGG binding site in vitro, correlated with a corresponding reduced DNA binding and transactivation in vivo.

The AMV-specific Mutations in R2 Alter the Relative Response to Subtypes of Myb-responsive Promoters in Vivo—We next asked whether the AMV-specific point mutations in R2 had any influence on the relative responses obtained with different subtypes of synthetic Myb-responsive promoters in vivo. For this purpose, we employed reporter constructs con-
containing three copies of MRE sequences having different configurations of Gs in positions 5 and 6 of the MRE consensus sequence; that is either 3×TAACGG, 3×TAACGT, or 3×TAACCT (designated "3×GG," "3×GT," or "3×TG reporters," respectively). A reporter with 3×TAACCT was used as negative control. As illustrated in Fig. 8A, the 3×GG reporter gave the highest levels of transactivation for both fusion proteins. The transactivation obtained with the 3×GT and 3×TG reporters were generally much lower, but still at a significant level when activated by the R2R3[wt]-VP16 fusion protein, consistent with our previous report (30). In contrast, the R2R3-[AMV]-VP16 fusion protein resulted in very low levels of transactivation for both the 3×GT and 3×TG reporters. This suggests that the reduced DNA binding seen with the R2R3[AMV] proteins, relative to the wild type protein, is more severe for certain MRE sequences than for others as already seen in in vitro data in Fig. 6. This is illustrated in Fig. 8B where the transactivation data for each effector are normalized to the 3×GG reporter. If the reduction in DNA binding caused by the AMV-specific point mutations were proportional for the different MREs, the three synthetic promoters analyzed would have given pairwise equal levels of transactivation when normalized as in Fig. 8B, which is clearly not the case. In particular the 3×TG-reporter responded very poorly to the R2R3[AMVNHD]-VP16 fusion protein, while it was reasonably activated by the R2R3[wt]-VP16 fusion protein.

Thus, we conclude that, even if the AMV-specific point mutations do not directly alter the sequence specificity of the protein as demonstrated previously (25), these mutations seem to weaken the interaction with subclasses of the recognition sequence to a different extent. It is therefore conceivable that different subsets of Myb-responsive promoters react differentially to these mutations and that these quantitative effects contribute to the altered patterns of gene activation induced by AMV v-Myb relative to wild type c-Myb.

**DISCUSSION**

In this work we have investigated how the properties of Myb are affected by the point mutations that have been selected for in the DNA binding domain of AMV v-Myb. Despite striking phenotypic effects in vivo (23, 24), the mechanism of action of these mutations has remained elusive since no change in sequence specificity of the mutated protein was observed (25), and no alterations in protein-protein interactions with a hypothetical partner have been reported. To better understand which properties of the protein might have been altered, we performed a detailed biochemical analysis of the minimal DNA binding domain in mutated versus wild type forms.

Most of the properties analyzed were indeed altered. The AMV-specific mutations in the second repeat had significant effects on conformation, redox-sensitivity, and on quantitative aspects of the DNA binding properties of the protein. Even if the mutated protein binds the same range of sequences as the wild type, we found a sequence dependence in the magnitude of destabilization of the complexes in vitro and in DNA binding in vivo. These latter observations in particular offer a possible explanation of how the AMV-specific mutations can lead to a different spectrum of genes activated by AMV-Myb versus c-Myb.

The evidence for a conformational change comes primarily from fluorescence quenching experiments, which showed that the average exposure of the many tryptophans in the DNA binding domain is reduced in the two R2R3[AMV] mutants compared to that in R2R3[wt]. Since the introduced AMV-specific mutations are located in R2, it is reasonable that the conformation of this domain was most affected. The R2 domain seems to be flexible and temperature-sensitive, and it changes conformation upon binding to DNA (15–17, 38). This conformational flexibility might well be sensitive to mutations. Previously analysis of Cys mutations located in R2 also changed the conformation of the domain. However, when we screened a series of 15 additional mutations distributed over the entire R2, none were found with the same properties as R2R3[AMV], suggesting that the AMV-specific mutations have a particular effect on the structure.

It is noteworthy that all three point mutations in R2 represent changes from rather hydrophobic residues to more polar ones (I91N, L106H, and V117D). Since they are all located on the surface of the protein (14), one possibility is that they might stabilize the folded structure of the domain through solvation effects. In addition, inspection of the structure reported by Ogata et al. (14) revealed that two of the mutations (L106H and V117D) were close to each other in space (Fig. 1B). If both were ionized, the resulting electrostatic interaction between His+ and Asp− could also have a stabilizing effect on the AMV v-Myb DNA binding domain. In accordance with this hypothesis all differences observed in this work could be attributed to the L106H and V117D mutations, since the two proteins R2R3[AMVNHD] and R2R3[AMVNHD] behaved equally in all comparative experiments.

R2 contains a single reactive cysteine that is exposed for modification in the free protein but protected in the tighter folded DNA-bound conformation (16). According to the structural model the cysteine is located in the interior of the protein in the DNA complex (14). The observation that the reactivity of this cysteine is reduced in the R2R3[AMV] proteins compared to the R2R3[wt] protein, suggests that the cysteine on average is in a less exposed conformation in R2R3[AMV]. It is possible that an unstable or nascent helix in the region around the cysteine (18) might be stabilized by a tighter conformation in other parts of R2. Alternatively, the reported cavity in R2 might be affected (17). The cysteine in R2 is highly conserved, but is not essential for DNA binding since hydrophobic substitutions may be introduced without reducing the DNA binding activity of the protein (16). Its high redox reactivity has led us and others to suggest that it might be conserved to keep the protein responsive toward a possible redox regulatory mechanism. The observation that the AMV-specific mutations make the protein less responsive toward Cys modification and oxidation suggests that the oncogenic version might remain partially active under conditions where the normal variant would be inactivated. This might contribute to its oncogenic potential under specific conditions.

Previous reports found no qualitative differences in DNA sequence recognition properties between AMV v-Myb and c-Myb (25). With respect to quantitative DNA binding properties AMV v-Myb subdomains were reported to bind stronger (19) or weaker (24) to MRE as judged by direct electrophoretic mobility shift assay using bacterial extracts or purified recombinant Myb. We therefore analyzed in more detail quantitative aspects of DNA binding using purified R2R3[wt], R2R3[AMVNHD], and R2R3[AMVNHD]. Although the three minimal DNA binding domains seemed very similar when analyzed in a direct mobility shift assay, competition assays revealed striking differences in stabilities between the formed complexes. Our analysis clearly demonstrates that the AMV-specific mutations weakens the interaction with the mim-1-derived recognition sequence. This adds to our previous hypothesis that R2 plays a role in modulation of complex stability. In keeping with that different variants of the second half-site bound by R2 lead to different complex stabilities (20), we show here that the AMV mutant versions of R2 also displays altered complex stability.

The most interesting effect of the AMV-specific mutations
was the differential destabilization on certain MRE variants. Binding to a TAACTG variant of MRE was much more destabilized than a complex with a TAACGG variant. The consequence of this differential destabilization when analyzed in an in vivo yeast system was that R2R3[AMVNHD]-VP16 still transactivated through a TAACGG variant of MRE, but gave very low level of transactivation through a TAACTG site. In contrast R2R3[wt]-VP16 transactivated both variants of synthetic promoters. Among the candidate target promoters for Myb reported in the literature, the TG variants of binding sites are found more often than the GG variants. It is therefore quite possible that certain subclasses of promoters activated by c-Myb will fall below a critical level of affinity and not be activated by AMV-v-Myb while other subclasses are activated by both factors. This may result in a different spectrum of genes activated by AMV-Myb relative to c-Myb.

Our analysis has revealed that several intrinsic properties of the minimal DNA binding domain of Myb are modified as a result of the AMV-specific mutations in R2. Even if we cannot directly link these changes to the observed phenotypic effects of the same mutations in transformed cells, it is quite probable that quantitative alterations in DNA binding properties will have important phenotypic effects. The prevailing alternative hypothesis has been to assume alterations in protein-protein interactions with a hypothetical partner. We cannot exclude that AMV-v-Myb later will prove also to have modified interactions with such partner proteins, but our results show that we do not need to postulate such alterations to explain phenotypic effects of the AMV point mutations. Alterations in other subdomains of the protein also mutated in AMV-v-Myb probably add to the changes analyzed in this work. It was recently reported that the transactivation properties of the protein was weakened as a result of some of these mutations (24, 39). Although it is intriguing that a more potent oncogene encodes a transcription factor with weakened DNA binding and reduced transactivation properties, the finding that most of the mutations have a clear effect probably reflects the long time through which these mutations have been selected for.

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