MDM2 proto-oncogene expression is aberrant in many human tumors. Its normal role is to modulate the functions of p53. The N terminus of MDM2 interacts with p53, whereas the properties of the rest of the molecule are poorly understood. We show that MDM2 binds to the general transcription factor TFIIID in vivo. The C-terminal Ring finger interacts with TAFII250/CCG1, and the central acidic domain interacts with TBP. Expression of MDM2 activates the cyclin A gene promoter but not c-fos, showing that the effects of MDM2 are specific. Deletion of the C-terminal region of MDM2 abolishes activation, showing that the C-terminal domain of MDM2 is functionally important. We found that increasing MDM2 expression to higher levels inhibits the cyclin A promoter. Inhibition appears to result from titration of general transcription factors because MDM2 overexpression inhibits c-fos as well as other promoters in vivo and basal transcription in vitro. The mechanisms of repression of the cyclin A and fos promoters appear to be different. Cyclin A repression is lost by deleting the C terminus, whereas that of c-fos is lost by removal of the acidic domain. These results reinforce the conclusion that the C terminus of MDM2 mediates effects on the cyclin A promoter. MDM2 transformed cells contain elevated levels of cyclin A mRNA, showing that activation occurs under physiological conditions. There is a positive correlation between MDM2 binding to TAFII250 and MDM2 activation of the cyclin A promoter. The C-terminal region of MDM2, which contains the Ring finger, interacts with TAFII250 and is required for regulation of the cyclin A promoter by MDM2. Our results link the activity of MDM2, a transforming protein implicated in many human tumors, with cyclin A, a regulator of the cell cycle.

The tumor suppressor p53 regulates the expression of downstream effectors involved in cell cycle arrest, recovery from the arrest, and apoptosis (1–3). The key mediators of these processes are, respectively, the cyclin-dependent kinase (cdk) inhibitor, p21Cip1/Waf1 (4), the proto-oncprotein MDM2 (mouse double minute 2) (5–10), and the cell death promoting factor Bax (11). MDM2 is aberrantly expressed in a number of human tumors (12–20). It forms a negative autoregulatory loop with p53 by binding to its activation domain (21) and inhibiting its functions in transactivation (22–24), growth arrest (25, 26), and apoptosis (26–28). Inhibition of p53 is essential for development, because homozygous inactivation of the MDM2 gene in mice is lethal and is rescued by inactivation of p53 (23, 29). MDM2 regulates a number of factors in addition to p53, including the tumor suppressor pRb (30), its homologue p107 (31), and the transcription factors E2F1/DP1 (32) and MyoD (33).

MDM2 has many features of transcription factors, including a nuclear localization signal, a central acidic region similar to a class of activation domains, an adjacent C4 zinc finger that could interact with nucleic acids, and a C-terminal variant C3HC4 Ring finger (34, 35). Ring fingers fold into a characteristic motif with two molecules of zinc and mediate interactions with nucleic acids and proteins (36, 37). The N-terminal region of MDM2 interacts with p53 (23, 29), E2F1/DP1 (32), and SV40 large T (38). Recently, the acidic region has been shown to interact with the ribosomal protein L5 and the Ring finger has been shown to interact with RNA, suggesting that MDM2 may regulate protein synthesis (39). The effects of MDM2 on p53, pRb, p107, and E2F1/DP1 suggest that one of its major functions is to regulate the cell cycle.

Cell cycle progression depends upon cyclin-cdk complexes that are regulated in a complex manner by synthesis and degradation of the cyclins, association with inhibitory subunits, and phosphorylation of activatory and inhibitory sites on the cdkks (40–43). Cyclin A is required for the S phase, passage through G2, and mitosis (44–48). Cyclin A synthesis at the G1/S border is tightly regulated at the transcriptional level (49), whereas degradation appears to be restricted to G2/M (50). The promotor is repressed during the G1 phase by factors binding the cell cycle-dependent element (CDE) and cell cycle genes homology region (CHR), and dissociation of these repressors appears to release the activity of positive regulators, leading to activation during S phase (49, 51–53). There are similar CDE/CHR elements in the cdc2 and CDC25C gene promoters that mediate regulation during the S/G2 phase (52), whereas related elements control G1 and G2/M phase expression (54). The factors that bind to the CDE and the CHR have not been unambiguously established. E2F/DP1-like factors appear to bind weakly to the CDE (51–53), and there is evidence that the
binding complex contains cyclin E, cdk2, p107, and an E2F-like factor (55). The association of E2F1 and p107 with both regulation of the cyclin A promoter and the activities of MDM2 suggests that cyclin A expression could be affected by MDM2.

We have found that MDM2 interacts physically with TAF\(_{2}^{250}\)/CCG1, a specific transcription accessory factor for the cyclin A promoter (56) that is required for G\(_{1}\) phase passage (57). MDM2 expression stimulates the cyclin A promoter. The C-terminal domain of MDM2, which interacts with TAF\(_{2}^{250}\), is required for activation of the promoter. Cyclin A expression is elevated in MDM2 transformed cells. These results provide a link between MDM2 and cyclin A, an important component of the cell cycle control machinery.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—GST fusion proteins were expressed in *Escherichia coli* BL21, grown at 28 °C, and purified on glutathione-Sepharose in the presence of 10 mM DTT as described previously (58). Insect cells (Sf9) were grown at 27 °C in Grace’s medium, infected with recombinant baculovirus producing MDM2, and harvested 72 h postinfection. The cells were pelleted, washed twice with ice-cold phosphate-buffered saline (1 mM Na\(_{2}\)HPO\(_{4}\), 10.5 mM KH\(_{2}\)PO\(_{4}\), 140 mM NaCl, pH 7.2), extracted with 3 mL of lysing buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 3 mM DTT, 0.35 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 2.5 μg/mL protease inhibitors), and incubated for 30 min on ice. Lysates were centrifuged at 30,000 rpm for 30 min. Clarified supernatants were incubated for 4 h at 4 °C with 1 mg of polyclonal antibody 365 cross-linked to protein A-Sepharose. The bound fractions were washed three times with 10 mL of ice-cold buffer A (20 mM HEPES, pH 8.0, 150 mM NaCl, 0.2% Tween 20, 5% glycerol, 2 mM DTT, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 2.5 μg/mL protease inhibitors). Proteins were eluted for 12 h at 4 °C in buffer A in the presence of 2 mg of the epitope peptide and concentrated on centricon-30. TFIID was purified by anion-exchange chromatography. Dual hybrid interaction assays were performed according to Vojtek and Hollenberg (64). To quantify β-galactosidase expression, yeast clones were grown in the appropriate selective media and assayed using ONPG. β-Galactosidase units are normalized to the amount of protein.

**Immunoprecipitation**—nuclear extracts were prepared from 2 × 10\(^{7}\) 3T3 cells at about 70% confluence as described by Shapiro et al. (68). The extract (a quarter per immunoprecipitation, 400 μl in 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 5% glycerol, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/mL protease inhibitors, 0.2% Tween 20, and 10 μg/mL bovine serum albumin) was incubated for 6 h at 4 °C with antibodies against TBP (3G3) and then for 2.5 h with protein A-Sepharose and washed three times with 1 mL of NET50 (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, and 0.25% gelatin), and the bound fractions were analyzed by 7% SDS-PAGE and Western blotting with MDM2 antibodies (365).

**RESULTS**

**MDM2 Interacts with TFIID in Vivo and in Vitro**—We investigated the possibility that MDM2 interacts with TFIID in vivo in 3T3 cells that are transformed by MDM2 (71). Crude extracts were immunoprecipitated with antibodies against TBP, a component of TFIID, and MDM2 was revealed by Western blotting. The immunoprecipitate contained specifically associated MDM2 (Fig. 1A, lane 2), which migrated at the expected molecular weight (90,000, compared with input in lane 1). MDM2 was not detected in controls lacking either antibodies or extract (lanes 3 and 4). Another component of the TFIID complex, TAF\(_{2}^{250}\), also co-immunoprecipitated (lane 6). TAF\(_{2}^{250}\) was detected with specific antibodies (lane 6), was migrated at the expected molecular weight (compare with input, lane 5),
The Oncogenic Co-activator MDM2

30653

Fig. 1. MDM2 interacts with TBP containing complexes. A, MDM2 forms a complex with TFIID in vivo. 3T3DM nuclear extracts (3T3DM N.E.) were immunoprecipitated with the TBP monoclonal antibody PAb3G3, transferred to nitrocellulose, and analyzed by Western blotting with the 2C1 TBP monoclonal antibody followed by autoradiography. A labeled protein of the expected size was specifically retained by MDM2 (lanes 1 and 2), Bound proteins were analyzed by SDS-PAGE followed by autoradiography. A labeled protein of the expected size was specifically retained by MDM2 (lanes 1 and 2, upper band labeled with an arrow), showing that the TFIID complex binds to MDM2. Several other bands were also detected, only one of which was specific (see lower band labeled with an arrow). The identity of this protein has not been established.

TBP Interacts with the Acidic Domain of MDM2—To study whether TBP associates with MDM2 in the absence of other components of TFIID, we used [35S]methionine-labeled proteins produced in E. coli. Human and yeast TBP were compared because they have highly related C-terminal domains. TFIIB was also studied because it is a basal transcription factor that mediates critical interactions with a number of activators (75, 76). Crude extracts from E. coli containing the overexpressed and other labeled proteins (Fig. 2A, lanes 1 and 4) were incubated with either GST or GST-MDM2. Human TBP was found to interact specifically with MDM2, as shown by both the amount retained on GST-MDM2 compared with GST (lanes 5 and 6) and the preferential retention over the labeled E. coli proteins. Yeast TBP also interacted specifically with MDM2, although with lower affinity than human TBP (data not shown), suggesting that the conserved domain mediates the interactions. TFIIB did not interact with MDM2 (lanes 1–3), providing further evidence for the specificity of the interaction with TBP and suggesting that TFIIB is not directly involved in regulation of transcription by MDM2.

Human TBP-MDM2 interactions were studied further using various combinations of in vitro translated proteins. MDM2 translated in vitro was retained specifically on GST-human TBP (Fig. 2B, lanes 1–6). Conversely, in vitro translated human TBP bound to GST-MDM2 (lanes 7–12), indicating that the nature of the fusion did not affect the interactions. A TBP-MDM2 complex was detected with purified proteins (results not shown). Taken together, these results show that the interactions are direct and are most probably not mediated through a protein bridge.

We used MDM2 deletion mutants to localize the sequences that interact with TBP. We initially established that the p53-binding domain is not required. MDM2 lacking the N-terminal domain interacted with GST-TBP (Fig. 2B, lanes 1–3), whereas this domain alone did not (MDM2(1–134), lanes 17–20). Starting from the p53-binding domain, the effect of progressively extending the sequences was studied. Elongation to amino acid 218 did not restore binding (MDM2(1–218), lanes 21–24); further addition progressively increased the interactions (MDM2(1–238), lanes 25–28; MDM2(1–265), lanes 29–32), attaining a maximum upon reaching 273 (MDM2(1–273), lanes 33–36). These results suggest that amino acids 219–273 mediate the interactions. Indeed, deleting this region in the full-length protein markedly decreased MDM2-TBP interactions (MDM2 ∆(219–273), lanes 37–40). C-terminal sequences do not mediate the residual interactions because additional deletion of amino acids beyond 304 had no further effect (MDM2(219–303), lanes 41–44). These results show that the acidic domain of MDM2 (219–273) contributes to interactions with TBP. As expected, the acidic domain alone was found to bind to TBP (results not shown). Interestingly, the MDM2 sequences from 245 to 253, within the acidic domain, are homologous to a motif found in a set of TATA-binding transactivators, including E1A, VP16, and...
c-fos (see underlined sequence in Fig. 2B) (77).

**MDM2 Interacts with TAFII250**—MDM2 and TAFII250 are implicated in cell cycle control, suggesting that their activities could be linked. We investigated whether TAFII250 and MDM2 interact physically using several in vivo and in vitro assays. Initially, interactions were studied by co-immunoprecipitation of MDM2 and hemagglutinin-tagged TAFII250 expressed by baculovirus in insect Sf9 cells. The cells were labeled with [35S]methionine, and extracts were precipitated with MDM2 antibodies (Fig. 3A). The MDM2 antibodies were initially shown to be specific. They precipitated MDM2 from cells co-infected with the MDM2 baculovirus (lane 4) and not TAFII250 from cells infected with the TAFII250 baculovirus (lane 3; compare with lane 1, input TAFII250, and lane 2, TAFII250 immunoprecipitated with anti-hemagglutinin). MDM2 was found to interact with TAFII250. MDM2 antibodies co-immunoprecipitated TAFII250 from co-infected cells expressing both proteins (lane 5).

The **MDM2 Ring Finger and the TAFII250 HMG-like Region Mediate Complex Formation**—We used GST assays to study the sequences of MDM2 that mediate interactions with TAFII250. [35S]Methionine-labeled TAFII250 extracted from baculovirus-infected Sf9 cells was fractionated on mini-columns containing immobilized GST fusion proteins. Full-length MDM2 fused to GST retained TAFII250 (lane 8), in contrast to GST alone (lane 6). The acidic domain (amino acids 221–272) of MDM2, which interacts with TBP (Fig. 2B), did not retain TAFII250 (Fig. 3A, lane 7). In contrast, the C-terminal Ring finger motif (amino acids 432–489) interacted specifically with TAFII250 (Fig. 3A, lane 9) with an efficiency similar to both full-length MDM2 (lane 8) and TBP (lane 10), which is known to form a strong complex with TAFII250. These results show that the Ring finger domain of MDM2 is sufficient to mediate efficient and specific interactions with TAFII250.

The TAFII250 sequence that interacts with MDM2 was localized by both in vivo and in vitro approaches. Initially we used the yeast dual hybrid system. TAFII250 fragments containing functional domains predicted by homology (66) were fused to the VP16 transactivation domain (Fig. 3B). They were tested for their ability to interact with MDM2 fused to the LexA DNA-binding domain by the β-galactosidase assay. Among the ten fragments tested, only the HMG homology region (construct 5) gave a significant and reproducible increase in β-galactosidase activity. The interactions were also studied in vitro using GST-MDM2, and TAFII250 fragments were synthesized in reticulocyte lysates (Fig. 3C). MDM2 interacted specifically with the HMG region (lanes 5 and 6), with about one-third the efficiency of the acidic domain of p53 (compare lanes 4 and 6 with lanes 13 and 15). In contrast, it did not interact with mutants that were negative in the yeast assay (SWI4, DR1, and acidic domains; lanes 1–3, 7–9, and 10–12). These results show that the TAFII250 HMG-like region interacts with MDM2. It also interacts with TFIIF (78) and E1A (79), raising the possibility that MDM2 might regulate transcription by affecting TAFII250-TFIIF interactions.

**MDM2 Activates the Cyclin A Promoter**—TAFII250 is specifically required for transcription from the cyclin A promoter (56). Because MDM2 interacts physically with TAFII250, we tested the possibility that it may regulate the cyclin A promoter using transient transfection assays. BHK cells were transfected with various amounts of an MDM2 expression vector (Fig. 4A) and a reporter containing 1,170 bp of the cyclin A promoter (Fig. 4B). MDM2 increased the promoter efficiency three-fold. The results show that the acidic domain of MDM2 is sufficient to mediate efficient and specific interactions with TAFII250.
promoter linked to luciferase coding sequences (49). The cells were transfected in high serum (10%), washed to remove the precipitate, and then incubated in low serum (0.07%) for 14 h. MDM2 expression stimulated cyclin A promoter activity about 4-fold at the optimum (0.25 μg vector) in three independent experiments. In these conditions the level of MDM2 expression was similar to that in 3T3DM cells that are transformed and overexpress MDM2 as determined by Western blotting and

Fig. 3. MDM2 interacts with TAF\(_{150}\). A, MDM2 interacts with TAF\(_{150}\) in vivo (lanes 1–5) and in vitro (lanes 6–10). Sf9 cells were infected with baculoviruses that express either hemagglutinin-tagged TAF\(_{150}\) (lanes 1–5, and 6–10), MDM2 (lane 4), or both (lane 5) and labeled with \[^{35}S\]methionine. Cleared lysates were either analyzed directly (lane 1) or immunoprecipitated with hemagglutinin tag antibodies (PAb12CA5; lane 2) or MDM2 polyclonal antibodies (lanes 3–5). Alternatively, extracts from cells expressing TAF\(_{150}\) were mixed with GST (lane 6), GST-AD (MDM2 221–272; lane 7), GST-MDM2 (lane 8), GST-RING (MDM2 432–489; lane 9) or GST-TBP (lane 10). The asterisk (lanes 1–5) indicates a nonspecific protein that is overexpressed with MDM2. B, the HMG-like domain of TAF\(_{150}\) mediates interactions with MDM2 in the yeast dual hybrid assay. Yeast cells were transformed with pLexA-MDM2 and TAF\(_{150}\)-VP16 fusion expression vectors, and β-galactosidase (βgal) activity was measured. Constructs 1–10 contain the following TAF\(_{150}\) sequences: 1, 1–198; 2, 166–980; 3, 957–1158; 4, 1132–1219; 5, 1190–1282; 6, 1254–1375; 7, 1351–1503; 8, 1473–1630; 9, 1610–1697; 10, 1671–1872. The expression in yeast cells of the various constructs was checked by Western blotting with an antibody against VP16. The diagram of TAF\(_{150}\) indicates the domains defined by homology (NFκB, SWI4, HMG, the direct repeats, the bromodomains, and the acidic region) (66, 79), the N and C-terminal kinase domains (74), the histone acetyl transferase domain (HAT) (108), the G residue that is mutated to D in tsBN462 (57), and the binding domains for TBP (109, 110) and RAP74 (74, 78). C, the HMG-like domain of TAF\(_{150}\) interacts with MDM2 in vitro. TAF\(_{150}\) mutants analyzed in the yeast dual hybrid system (construct 3, lanes 1–3; construct 5, lanes 4–6; construct 7, lanes 7–9; construct 10, lanes 10–12) and p53 (amino acids 1–52; lanes 13–15) were translated in reticulocyte lysate and either analyzed directly (Load) or fractionated on GST or GST-MDM2 as indicated. The asterisk indicates a nonspecific protein labeled during translation.
correction for transfection efficiency (data not shown and see below). Activation was lower with the highest amount of expression vector (1 μg), suggesting that MDM2 overexpression titrates a limiting factor (see below). The activity of the c-fos promoter is not affected by a mutation in TAFII250 (56), raising the possibility that MDM2 expression would not affect its activity. We co-transfected a c-fos promoter-CAT reporter together with the cyclin A luciferase reporter. MDM2 expression had no effect on c-fos promoter activity with levels of MDM2 expression vector that maximally stimulated the co-transfected cyclin A promoter (Fig. 4B, 0.25 μg of MDM2). However, the c-fos promoter was inhibited with the highest amount of MDM2 expression vector, again suggesting that MDM2 overexpression titrates a limiting factor. These results suggest that MDM2-TAFII250 interactions may lead to specific activation of the cyclin A promoter.

The C Terminus and the Nuclear Localization Signal of MDM2 Are Required for Activation of the Cyclin A Promoter—We tested whether the MDM2 sequences that mediate interactions with TAFII250 are required for activation of the cyclin A promoter (Fig. 5A). Mutant 1, lacking 30 amino acids from the C terminus, did not activate (Fig. 5A), even though it was expressed in similar amounts as the wild type protein, as determined by Western blotting of cell extracts with MDM2 antibodies (Fig. 5B, lanes 1–3). Mutant 1 was inactive, even when the quantity of expression vector was increased (up to 5 μg; data not shown). Mutants 2 and 3, with further deletions from the C terminus, were also inactive (Fig. 5A) and were expressed at levels comparable with that of the wild type protein (Fig. 5B, lanes 4–7). These results show that the C-terminal region (amino acids 390–489) is required for cyclin A promoter activity. Because transcription occurs in the nucleus, we investigated the effect of deletion of the nuclear localization signal. Mutant 4 that lacks the nuclear localization signal was inactive (Fig. 5A), cytoplasmic (as determined by immunocytofluorescence; data not shown), and expressed at similar levels as the full-length protein (Fig. 5B, lanes 8–10). These results show that MDM2 acts in the nucleus, in agreement with the possibility that it could affect activation by MDM2. We found that high serum stimulates the transfected cyclin A promoter about 20-fold (data not shown) and abrogated MDM2 activation with levels of expression vector that were optimal under low serum condition (Fig. 6A, see 0, 0.05, and 0.25 μg of expression vector and compare with Fig. 4A). These results suggest that MDM2 and serum activate the cyclin A promoter through the same pathways. To test this hypothesis directly we investigated whether mutation of the CDE would affect activation by MDM2 in low serum. The transfections contained reporters with cyclin A promoter.

Fig. 4. MDM2 specifically activates the cyclin A promoter in BHK21 cells. BHK21 cells were transiently transfected in high serum with various amounts of a CMV vector that expresses full-length MDM2 and two reporters, a luciferase reporter with 1,170 bp of the cyclin A promoter (1.5 μg (49) and a CAT reporter with 400 bp of the c-fos (1.5 μg) (111). The cells were incubated in low serum (0.07% fetal calf serum), and luciferase (A) and CAT (B) activities were measured 48 h later. FOLD:CAT, activities relative to 0 μg of the MDM2 expression vector.

Fig. 5. Effects of MDM2 mutants on the activity of the cyclin A promoter. A, tsBN462 cells were transfected with expression vectors (1 μg) for the indicated mutants and the cyclin A promoter-luciferase reporter, incubated at 39 °C in low serum, and analyzed for luciferase expression. The expressed MDM2 proteins have the following sequences or deletions (Δ): wild type (wt), 1–499; mutant (mut) 1, 1–460; mutant 2, 1–273; mutant 3, 1–218; mutant 4, Δ154–221. B, extracts of cells transfected with the indicated expression vectors were electrophoresed on either 7% (lanes 1–4 and 8–10) or 15% (lanes 5–7) polyacrylamide-SDS gels and analyzed by Western blotting with polyclonal MDM2 antibodies (PAb 365, lanes 1–7; PAb 370, lanes 8–10; see “Experimental Procedures”).
A promoter sequences from 2lyzed for luciferase expression.

MDM2 expression vector, incubated at 39 °C in low serum, and ana-
fors basal transcription, raising the possibility that MDM2 over-
scription in vitro (Fig. 7). We tested this possibility using mutants of MDM2 that affect interactions with TBP and TAF1250. BHK cells growing asynchronously in high serum were co-transfected with large amounts (5 µg) of expression vectors and the cyclin A-luciferase and fos-CAT reporters, and extracts were assayed for both luciferase and CAT activity. Mutant 1 with a small deletion of C-terminal sequences (amino acids 461–489) repressed both promoters (Fig. 7C). Mutant 2, which lacks the whole of the C-terminal region, was substan-
tially inactivated toward inhibition of the cyclin A promoter but still efficiently inhibited c-fos (Fig. 7C). Mutant 3, which lacks in addition the acidic domain, no longer repressed c-fos. These results show that the cyclin A and c-fos promoters respond differently to mutation of MDM2. They suggest that interac-
tions of the C terminus with TAF1250 and the acidic domain with TBP mediate repression. The results with mutant 1 are not necessarily incompatible with these conclusions. Mutant 1 has lost the ability to activate the cyclin A promoter (Fig. 5A) but not repression. It may lack only part of a larger functional domain, because a mutant with a deletion of the adjacent region (amino acids 365–426) did not activate the cyclin A promoter (data not shown). The small C-terminal deletion may only partially affect interactions, and in excess it may still titrate a limiting factor.

**Elevated Levels of Cyclin A mRNA in MDM2 Transformed Cell Lines**—The ability of MDM2 to activate the cyclin A promoter in transfection assays raised the possibility that cyclin A levels may be elevated in MDM2 transformed cells. We ana-
yzed cyclin A gene expression in several MDM2 transformed cell lines. 3T3DM cells are spontaneously transformed fibro-
blasts that have multiple copies of the mdm2 gene and express high levels of MDM2 (71). N/mmdm2 cells contain a mdm2 gene that was introduced on a cosmid and express lower levels of MDM2. N/pCV001 are the equivalent control cells that contain the empty vector (34). Cyclin A RNA was analyzed by both Northern blotting (Fig. 8A) and semi-quantitative PCR (Fig. 8B), using as internal controls either ribosomal RNA (Fig. 8A, lanes 1–3) or glucose-6-phosphate dehydrogenase (Fig. 8B, lanes 1–3). We found in serum deprived cells that there are elevated levels of cyclin A RNA in both MDM2 transformed cell lines and especially in 3T3DM cells, which express a greater amount of MDM2 (Fig. 8, A, lanes 4–6, and B, lanes 1–3). These results suggest that one of the consequences of transformation by MDM2 is an increase in cyclin A gene expression.

**DISCUSSION**

**MDM2 Interacts with TBP and TAF1250**—We have found that MDM2 interacts in vivo and in vitro with the general transcription factor TFIIID. In contrast, MDM2 does not bind to TFIIH, a general factor that interacts with a number of activators and co-activators (75, 76, 82–84). Our in vivo and in vitro studies show that two different domains of MDM2 contact two subunits of the complex, the acidic domain TBP and the Ring finger TAF1250. Importantly, these interaction domains lie in a poorly characterized part of MDM2, C-terminal to the p53-binding domain.

We detect MDM2-TBP interactions in a number of ways. MDM2 is present in complexes immunoprecipitated from

---

**Fig. 6.** MDM2 activation of the cyclin A promoter is abrogated by high serum and mutation of the CDE. A, effect of high serum on activation by MDM2. Unsynchronized BHK21 cells cultured in high serum (10% fetal calf serum) were transfected with increasing amounts of the MDM2 expression vector and the cyclin A-luciferase reporter, and luciferase activity was measured after 24 h. B, effect of CDE mutation on activation in low serum. tsBN462 cells were transfected with cyclin A reporters containing either wild type (black bars) or mutated CDE (white bars; −214 to +100; Ref. 52) and various amounts of the pXJ41 MDM2 expression vector, incubated at 39 °C in low serum, and ana-
yzed for luciferase expression.

RNA polymerase II, as shown by rescue of heat-inactivated extracts with purified TFIIID (lanes 3 and 9) and by inhibition with α-amanitin (lane 2). These results indicate that MDM2 inhibits through interactions with general transcription factors.

**Different Sequences of MDM2 Are Required for Inhibition of the Cyclin A and c-fos Promoters**—MDM2 inhibition is ob-
erved with both the cyclin A and c-fos promoters, whereas activation is specific for cyclin A, suggesting that different factors are involved in both processes. We tested this possibility with mutants of MDM2 that affect interactions with TBP and TAF1250. BHK cells growing asynchronously in high serum were co-transfected with large amounts (5 µg) of expression vectors and the cyclin A-luciferase and fos-CAT reporters, and extracts were assayed for both luciferase and CAT activity. Mutant 1 with a small deletion of C-terminal sequences (amino acids 461–489) repressed both promoters (Fig. 7C). Mutant 2, which lacks the whole of the C-terminal region, was substan-
tially inactivated toward inhibition of the cyclin A promoter but still efficiently inhibited c-fos (Fig. 7C). Mutant 3, which lacks in addition the acidic domain, no longer repressed c-fos. These results show that the cyclin A and c-fos promoters respond differently to mutation of MDM2. They suggest that interac-
tions of the C terminus with TAF1250 and the acidic domain with TBP mediate repression. The results with mutant 1 are not necessarily incompatible with these conclusions. Mutant 1 has lost the ability to activate the cyclin A promoter (Fig. 5A) but not repression. It may lack only part of a larger functional domain, because a mutant with a deletion of the adjacent region (amino acids 365–426) did not activate the cyclin A promoter (data not shown). The small C-terminal deletion may only partially affect interactions, and in excess it may still titrate a limiting factor.

**Elevated Levels of Cyclin A mRNA in MDM2 Transformed Cell Lines**—The ability of MDM2 to activate the cyclin A promoter in transfection assays raised the possibility that cyclin A levels may be elevated in MDM2 transformed cells. We ana-
yzed cyclin A gene expression in several MDM2 transformed cell lines. 3T3DM cells are spontaneously transformed fibro-
blasts that have multiple copies of the mdm2 gene and express high levels of MDM2 (71). N/mmdm2 cells contain a mdm2 gene that was introduced on a cosmid and express lower levels of MDM2. N/pCV001 are the equivalent control cells that contain the empty vector (34). Cyclin A RNA was analyzed by both Northern blotting (Fig. 8A) and semi-quantitative PCR (Fig. 8B), using as internal controls either ribosomal RNA (Fig. 8A, lanes 1–3) or glucose-6-phosphate dehydrogenase (Fig. 8B, lanes 1–3). We found in serum deprived cells that there are elevated levels of cyclin A RNA in both MDM2 transformed cell lines and especially in 3T3DM cells, which express a greater amount of MDM2 (Fig. 8, A, lanes 4–6, and B, lanes 1–3). These results suggest that one of the consequences of transformation by MDM2 is an increase in cyclin A gene expression.

**DISCUSSION**

**MDM2 Interacts with TBP and TAF1250**—We have found that MDM2 interacts in vivo and in vitro with the general transcription factor TFIIID. In contrast, MDM2 does not bind to TFIIH, a general factor that interacts with a number of activators and co-activators (75, 76, 82–84). Our in vivo and in vitro studies show that two different domains of MDM2 contact two subunits of the complex, the acidic domain TBP and the Ring finger TAF1250. Importantly, these interaction domains lie in a poorly characterized part of MDM2, C-terminal to the p53-binding domain.

We detect MDM2-TBP interactions in a number of ways. MDM2 is present in complexes immunoprecipitated from
MDM2 expression vectors (5 μg) infected insect cells prepared by the protocol (lane 8 without increasing amounts of immunopurified MDM2 (AdMLP containing the adenovirus major late promoter (59, 87–89). The TBP is a component of TFIID, suggesting that MDM2 is involved in transcription mediated by RNA polymerase II (85, 86) and in specific functions of RNA polymerase II (59, 87–89). The TBP complexes with different transcription accessory factors that have specific functions. These complexes are involved in transcription by the three RNA polymerases (85, 86) and in specific functions of RNA polymerase II (59, 87–89). The TBP complexes isolated from both 3T3DM and HeLa extracts contain TAFII250, a component of TFIID, suggesting that MDM2 is involved in transcription mediated by RNA polymerase II.

However, we cannot exclude the possibility that MDM2 also associates with other TBP complexes. MDM2 binds to isolated subunits of TFIID, suggesting that it interacts directly with TFIID, rather than indirectly through other factors. MDM2-TFIID complexes are present in transformed 3T3DM cells that overexpress MDM2 (34, 71). It remains to be seen if TFIID-MDM2 interactions are important for transformation.

There are at least three distinct interaction domains in MDM2. The central region mediates interactions with TBP, because N- and C-terminal sequences (1–154 and 305–491) can be deleted without affecting binding (see "Results"). The major component of the interaction domain (219–273) is within the acidic region (220–300). It extends around a sequence (underlined in Fig. 2B) that resembles the TBP-binding motif of c-fos, VP16, and EIA (77) (VEVEVESLD in MDM2, SSFVFTYPE in c-fos, EEFVLYYVE in EIA, and DDDFLDLMG in VP16, where acidic or identical amino acid in at least two of the sequences are underlined). The central region also binds the L5 ribosomal protein (39).

The C-terminal Ring finger region of MDM2 binds to TAF1250 with similar efficiency as full-length MDM2, whereas the acidic region does not interact. The affinity is comparable to that of TBP, a well established interacting protein. The structures of several Ring fingers have been reported (90, 91). MDM2 has a variant Ring finger motif, with a threonine in the place of the third cysteine of the C3HC4 motif. This could be a conservative substitution of a hydroxyl group for a sulfydryl that coordinates zinc (35). It remains to be seen if the Ring finger structure is important for the interaction of MDM2 with TAF1250. Interestingly, the MDM2 Ring finger has recently been shown to interact specifically with RNA (39). Ring fingers were originally proposed to be nucleic acid-binding structures and have been shown to interact with nucleic acids. These interactions may be favored by the high positive charge of the surface of the Ring domain (37). More recently, Ring finger
containing proteins have been proposed to have an important role in forming large multi-protein complexes, such as the promyelocytic leukemia nuclear bodies. Ring fingers may participate in protein-protein interactions that act as a glue for assembly (37). However, MDM2 has not been described to form large assemblies, pointing to a different role for the MDM2 Ring finger. Elenbaas et al. (39) have proposed that the ability of MDM2 to contact both the L5 ribosomal protein and RNA through separate domains may indicate a role for MDM2 in the cytoplasm in regulating translation. Interestingly, the ability of the same two regions to contact two subunits of TFIID point to a nuclear function of MDM2 in regulating transcription.

The N-terminal domain of MDM2 has been most extensively studied. It is highly conserved across species and interacts with a number of proteins, most notably p53. The structure of the N-terminal region complexed to the activation domain of p53 has been determined (21). The binding domain englobes amino acids 17–125 and consists of a repetition of a \( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \alpha \)-\( \a
A is required for the G/S transition and passage through S and G$_2$ (44, 46, 47). Transcriptional regulation of cyclin A expression is important for S phase entry (49) and is the target of many cellular signals and factors, such as cell adhesion, cAMP, TGF-$\beta$, p53, p107, cyclin E, p27$^{kip1}$, and Myc (55, 81, 93, 96–101). It is also targeted by the viral oncoproteins HPV 16 E7, adenovirus EIA, and SV40 T (94–96). Cyclin A overexpression advances S phase entry (48, 102) and confers anchorage-independent growth (97), whereas loss of cyclin A results in early embryonic lethality (103).

MDM2 levels are principally regulated by p53 in response to DNA damage (7, 9, 27). MDM2 induction is a late response that follows p21$^{cip1/waf1}$, the major mediator of p53-induced G$_1$ cell cycle arrest (4, 104, 105). Induction of MDM2 correlates with recovery of normal DNA synthesis and may serve either to signal successful repair or to limit the severity or length of the arrest (7, 98). MDM2 inhibits both the transcriptional and apoptotic functions of p53. Diminishing the levels of MDM2 in response to DNA damage by cisplatin induces apoptosis (27). MDM2 induction is a late response that occurs following recovery from DNA damage (7, 9, 27). MDM2 induction is a late response that follows p21$^{cip1/waf1}$, the major mediator of p53-induced G$_1$ cell cycle arrest (4, 104, 105). Induction of MDM2 correlates with recovery of normal DNA synthesis and may serve either to signal successful repair or to limit the severity or length of the arrest (7, 98). MDM2 inhibits both the transcriptional and apoptotic functions of p53. Diminishing the levels of MDM2 in response to DNA damage by cisplatin induces apoptosis (27).

A is required for the G/S transition and passage through S and G$_2$ (44, 46, 47). Transcriptional regulation of cyclin A expression is important for S phase entry (49) and is the target of many cellular signals and factors, such as cell adhesion, cAMP, TGF-$\beta$, p53, p107, cyclin E, p27$^{kip1}$, and Myc (55, 81, 93, 96–101). It is also targeted by the viral oncoproteins HPV 16 E7, adenovirus EIA, and SV40 T (94–96). Cyclin A overexpression advances S phase entry (48, 102) and confers anchorage-independent growth (97), whereas loss of cyclin A results in early embryonic lethality (103).

MDM2 levels are principally regulated by p53 in response to DNA damage (7, 9, 27). MDM2 induction is a late response that follows p21$^{cip1/waf1}$, the major mediator of p53-induced G$_1$ cell cycle arrest (4, 104, 105). Induction of MDM2 correlates with recovery of normal DNA synthesis and may serve either to signal successful repair or to limit the severity or length of the arrest (7, 98). MDM2 inhibits both the transcriptional and apoptotic functions of p53. Diminishing the levels of MDM2 in response to DNA damage by cisplatin induces apoptosis (27). MDM2 induction is a late response that follows p21$^{cip1/waf1}$, the major mediator of p53-induced G$_1$ cell cycle arrest (4, 104, 105). Induction of MDM2 correlates with recovery of normal DNA synthesis and may serve either to signal successful repair or to limit the severity or length of the arrest (7, 98). MDM2 inhibits both the transcriptional and apoptotic functions of p53. Diminishing the levels of MDM2 in response to DNA damage by cisplatin induces apoptosis (27).
