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# Retinoic Acid Receptors Interact Physically and Functionally with the T:G Mismatch-specific Thymine-DNA Glycosylase\*

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The pleiotropic effects of retinoids are mediated by nuclear receptors that are activated by 9-*cis*- or all-*trans*-retinoic acid to function as ligand-dependent transcription factors. In a yeast one-hybrid screen for proteins capable of interacting with native retinoic acid receptor (RAR), we have isolated the T:G mismatch-specific thymine-DNA glycosylase (TDG), which initiates the repair of T:G mismatches caused by spontaneous deamination of methylated cytosines. Here, we report that TDG can interact with RAR and the retinoid X receptor (RXR) in a ligand-independent manner, both in yeast and *in vitro*. Mapping of the binding sites revealed interaction with a region of the ligand binding domain harboring  $\alpha$ -helix 1 in both RAR and RXR. In transient transfection experiments, TDG potentiated transactivation by RXR from a direct repeat element spaced by one nucleotide (DR1) and by RXR/RAR heterodimers from a direct repeat element spaced by five nucleotides (DR5). *In vitro*, TDG enhanced RXR and RXR/RAR binding to their response elements. These data indicate that TDG is not only a repair enzyme, but could also function in the control of transcription.

Retinoic acids exert their pleiotropic effects on vertebrate development and homeostasis by binding to nuclear receptors that function as ligand-dependent transcription factors regulating the expression of target genes. These receptors belong to a gene superfamily including the receptors for steroid hormones, thyroid hormone, vitamin D3, and a growing number of the so-called orphan receptors for which no ligands have yet been found (1–3). The receptors for retinoic acids comprise two

families, the retinoic acid receptors (RARs)<sup>1</sup> and the retinoid X receptors (RXRs), each consisting of three isotypes ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) (4). RARs bind all-*trans*-retinoic acid (t-RA) and 9-*cis*-retinoic acid (9c-RA), whereas RXRs respond exclusively to 9c-RA. As all nuclear receptors, they share a conserved modular structure with five (RXRs) or six regions (RARs) denoted as A to E or A to F, respectively. Out of these, the central C region harboring the core of the DNA-binding domain shows the highest homology among all receptors. The C-terminal E region contains the ligand binding domain (LBD), which functions as a ligand-dependent transactivation domain (AF-2), and contains surfaces for the homo- and heterodimerization with other receptors and interaction with various cofactors. The ligand-dependent activation domain AF-2 in the E region can synergize with the ligand-independent activation domain AF-1 located in the N-terminal A/B region. RARs and RXRs bind as dimers to their response elements (RAREs) consisting of two hexameric motifs (PuG(G/A)(T/A)CA) usually arranged as direct repeats. Although RXRs can bind on their own as homodimers to direct repeat elements spaced by one nucleotide (DR1), response elements for RXR/RAR heterodimers are direct repeats with a spacing of one (DR1), two (DR2), or five nucleotides (DR5) (4). After binding to their response elements they modulate transcription in a promoter-specific manner by acting on the transcription machinery or through remodeling of the chromatin template. The link to the basic transcription factors and proteins involved in chromatin remodeling is most likely mediated by direct protein-protein interactions with cofactors (3, 5, 6). Recently, several such proteins were cloned and characterized as putative corepressors like N-CoR (7) and SMRT (8) or coactivators like Trip1/Sug1 (9, 10), TIF1 $\alpha$  (11), RIP140 (12), SRC-1 (13), TIF2/GRIP1 (14–17), AIB1/RAC3/ACTR/p/CIP (18–21), and CBP/p300 (22, 23) which bind to RAR and/or RXR in a ligand-regulated manner. In addition to these interactions with different cofactors, the AF-1 function of RAR can be stimulated through binding to the general transcription factor TFIIF and phosphorylation by Cdk7 (24, 25).

Using a one-hybrid screening in yeast for putative cofactors of RAR, we report here the isolation of a new splice variant of the T:G mismatch-specific thymine-DNA glycosylase (TDG), which is required for the repair of methylated DNA sites. In vertebrates, DNA is most commonly modified by addition of a methyl group to the carbon 5 position in the pyrimidine ring of cytosines. Changes in 5-methylcytosine methylation patterns

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank<sup>TM</sup>/EBI Data Bank with accession number(s) AF069519.

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<sup>1</sup> The abbreviations used are: RAR, retinoic acid receptor; RXR, retinoid X receptor; TDG, thymine-DNA glycosylase; t-RA, all-*trans*-retinoic acid; 9c-RA, 9-*cis*-retinoic acid; AAD, acidic activation domain; LBD, ligand binding domain; AP, abasic site; RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair(s); tk, thymidine kinase; GST, glutathione S-transferase; EMSA, electrophoretic mobility shift assay; CIP, calf thymus intestinal phosphatase.

have been implicated in various processes such as control of gene expression (26), chromatin structure (27), somatic X-chromosomal inactivation in females (28), timing of DNA replication (29), and genomic imprinting (30). Methylation of cytosines is essential in vertebrates (31). However, it also causes genome instability; although not more than 1% of bases are methylated cytosine in mammalian genomes, they probably cause one third of all transition mutations responsible for genetic diseases and cancer in humans (32–34). This is due to the much higher spontaneous deamination of 5-methylcytosine when compared with cytosine; deamination of 5-methylcytosine leads to T:G mismatches, whereas deamination of cytosine generates U:G mismatches in the DNA. The repair of U:G and T:G DNA mismatches is initiated by mismatch-specific DNA glycosylases (35), which excise in a first and rate-limiting step the mismatched base leaving an abasic site (AP) that is cleaved by an AP-endonuclease and deoxyphosphodiesterase to create a single nucleotide gap; this gap is then filled in by DNA polymerase- $\beta$  and finally sealed by DNA ligase (36). TDG was first characterized as a 55 kDa protein purified from HeLa cells (37). Further analysis revealed that TDG was not only able to excise thymine from T:G, but also uracil from U:G DNA mismatches (38). The cloning of the human TDG cDNA showed that this glycosylase belongs to a new class of excision repair enzymes with no significant sequence similarity to the established DNA glycosylase gene family including the U:G mismatch-specific uracil-DNA glycosylase (UNG) (39). However, the gene for human TDG contains conserved regions with greater than 30% sequence identity with an *E. coli* gene (MUG) which was found later to encode an U:G mismatch-specific DNA glycosylase that, in contrast to the human TDG, cannot cut at T:G mismatches under physiological conditions (40). The recently published crystal structure of the *E. coli* MUG, nevertheless, revealed structural and functional homologies with UNG despite of their low sequence similarity (41). Interestingly, TDG was initially isolated and characterized in a yeast two-hybrid screening as a protein that can interact with the transcription factor c-Jun (42). This interaction, together with those with RAR and RXR described here points to a possible function of TDG in transcription beyond its role in maintenance of methylated CpG sites in DNA, and provides a further link coupling transcription and DNA repair.

#### EXPERIMENTAL PROCEDURES

**Cloning of mTDG Isoforms**—The *Saccharomyces cerevisiae* reporter strain BP-G5 (MAT $\alpha$  *ura3- $\Delta$ 1 his3- $\Delta$ 200 leu2- $\Delta$ 1 trp1::DR5 RARE-URA3) was derived from the strain PL1 (MAT $\alpha$  *ura3- $\Delta$ 1 his3- $\Delta$ 200 leu2- $\Delta$ 1 trp1::ERE-URA3) (43) by substituting the ERE by one copy of a DR5 RARE (AGG TCAGcgagAGG TCA). The one-hybrid screening was performed as described in Ref. 10 using *S. cerevisiae* strain BP-G5 expressing full-length hRAR $\alpha$ 1 from YE90, and a VP16-tagged mouse embryo cDNA library (10). Library plasmids from positive clones were isolated and sequenced leading to the isolation of a TDG cDNA encoding the TDGb isoform. A cDNA fragment (286–2567 bp) was used as a probe to screen a mouse embryo cDNA library (10 days post coitum) for additional TDG cDNAs. A full-length mTDGa cDNA was cloned by PCR using a 5'-primer encoding the missing 8 amino acids (ATG GAC GCG GAG GCC GCG AGA AGC) and the mTDGb cDNA as a template.**

**Plasmid Constructs**—For the expression of full-length or various mTDG fragments, the pSG5 vector (44) was used for mammalian cells, the pET15b vector (Novagen) for His-tagged proteins in *Escherichia coli*, and the pERV vector<sup>2</sup> for expression from Vaccinia virus. Reporter gene constructs and expression vectors for RXR and RAR were as described (47–49); Gal4-c-Jun fusion proteins were expressed from a pG4MpolyII vector (45), and other Gal4 fusion proteins were as described (46). Further details on all constructs used in this study are available on request.

**Northern Blots and RT-PCR**—Northern blot analyses were per-

formed using a mouse multi-tissue mRNA blot (CLONTECH) and a <sup>32</sup>P-labeled fragment of the mTDGb cDNA (286–2567 bp) as described by the manufacturer. RNA preparation from P19 cells and RT-PCRs were as described (47) using the following primers: TDGa-specific 5'-primer: GAG ACC GGC TGC CCG TGT GCC (bp 343–363 in hTDGa cDNA; Ref. 39), TDGb-specific 5'-primer: AGC AGC GTG GGA GGG GCC GAG (bp 1–21 in mTDGb cDNA), common 3'-primer: CCA GGC CCA GGG TAG TGA TGT CC (bp 514–536 in mTDGb cDNA).

**Antibodies and Western Blotting**—Anti-TDG monoclonal antibodies were raised against *E. coli*-expressed mTDGa(32–421) purified on a nickel matrix as described in Ref. 48. Western blots were performed according to standard protocols using 50  $\mu$ g of protein from whole cell extracts separated on 10% SDS gels. For dephosphorylation experiments, 25  $\mu$ g of a whole cell extract from MCF7 cells were incubated for 30 min at 37 °C with 20 units of calf thymus intestinal phosphatase (CIP) in a buffer provided by the manufacturer (Boehringer Mannheim). To avoid nonspecific degradation, leupeptin, pepstatin, aprotinin, antipain, and chymostatin were added to the reactions at a final concentration of 2.5  $\mu$ g/ml, and vanadate was used at a concentration of 50  $\mu$ M.

**Two-hybrid System and Transient Transfections**—For the mapping of the interaction domains in yeast, the LexA system was applied using the vectors pBTM116 (49) for the cloning of the LexA fusions and pASV3 (50) for the cloning of the VP16 fusions.  $\beta$ -Galactosidase assays on individual L40 transformants were carried out as described (46). The expression of all LexA and VP16 fusion proteins was checked by Western blotting using anti-LexA or anti-VP16-specific antibodies (data not shown). Transient transfections in COS cells as well as CAT and  $\beta$ -galactosidase assays were done as described in Ref. 51. All transfections employed duplicate samples, and were repeated at least twice. Representative experiments are shown in the figures.

**In Vitro Assays**—*In vitro* binding studies using GST-tagged hRAR $\alpha$  and mRXR $\alpha$  were carried out as described (11, 52). Electrophoresis mobility shift assays (EMSAs) were performed using *E. coli*-expressed RARAAB, RXRAAB, Vaccinia virus expressed mTDGa(32–421), and <sup>32</sup>P-labeled double-stranded oligonucleotides (DR1: 5'-GGG TCA A AGG TCA-3', DR5: 5'-AGG TCA agcttc AGG TCA-3'). The binding reaction was done in the presence of 1  $\mu$ g of bovine serum albumin and protein-DNA complexes and free DNA were then submitted to non-denaturing gel electrophoresis as described (53).

#### RESULTS

**Isolation and Characterization of a New Splice Variant of TDG**—Previous studies in yeast have shown that RAR cannot efficiently induce transcription from a DR5 element without heterodimerization with RXR (54–56). In a search for additional partners allowing RAR to transactivate from such an element, the yeast strain BP-G5 harboring one DR5 element within the URA3 promoter was designed for a functional one-hybrid screening with full-length RAR $\alpha$  as a bait and a mouse embryo (9.5 to 12.5 days post coitum) cDNA library fused to the acidic activation domain of the viral VP16 protein. Positive clones from this screening were expected not only to interact with RAR, but also to stabilize its binding to the DR5 element, thus allowing efficient RAR-mediated activation of the selection marker. Out of 30 positive clones obtained from the screening, 25 were identified as RXRs. From the five remaining clones, three encoded for another nuclear receptor, COUP-TFI (57), and two for TDG, which was initially cloned as a c-Jun-interacting protein (42). The 2859-bp TDG cDNA clone obtained from the yeast screening (denoted b isoform, Fig. 1, A and B) differs between positions 1 and 58 from the previously published sequences of TDG, here called TDG isoform a (39). Due to these differences, the mouse TDGb cDNA lacks the codon for the starting methionine of the TDGa isoform and encodes for a truncated form of the enzyme missing the first 25 amino acids (Fig. 1B). Inasmuch as, in the TDGb cDNA, no stop codon was found in front of the first methionine (Fig. 1A), a mouse embryo (10 days post coitum) cDNA library was screened for longer mTDG cDNAs. However, this screening did not result in the cloning of further 5'-extended TDGb cDNA.

The expression of the mouse TDGa and TDGb mRNAs was confirmed by RT-PCR analysis performed with isoform-specific

<sup>2</sup> E. Remboutsika, unpublished data.

## A

AGC AGC GTG GGA GGG GCC GAG GTC AGC GCC GTC CAT CCA CCG GTC GCC CCG ATC CTG TGC 60  
s s v g g a e v s a v h p p v a p i l c  
TAT TCT CTG GAG CAA GTT CAA GCT TTG TAT TCA TTT CCA TTT CAA CAA ATG ATG GCA GAA 120  
y s l e q v q a l y s f p f q q M M A E (4)  
GTT CCT AAC ATG GCA GTC ACT ACT GGA CAG CAG GTG CCA GCA GAT GCT CCT AAC ATG GCA 180  
v p n m a v t t g q q v p a v a p n m a (24)  
ACC GTG ACA GAA CAG CAG GTG CCC GCA GAC GCT CCT GTC CAG GAA CCT GCA CCA GAA GCT 240  
T V T E Q Q V P A D A P V Q E P A P E A (44)  
CCA AAG AGA AGG AAA AGG AAA CCC AGA GCA GCA GAG CCC CAG GAA CCA GTG GAG CCC AAA 300  
P K R R K R K R K P R A A E P Q E P V E P K (64)  
AAA CCT GCT ACG TCG AAG AAA TCC GGC AAG TCT ACA AAA TCA AAG GAA AAG CAG GAG AAA 360  
K P A T S K K S G K S T K S K E K Q E K (84)  
ATC ACA GAC GCG TTT AAA GTG AAA AGG AAA GTG GAC CGC TTC AAC GGC GTC TCT GAA GCT 420  
I T D A F K V K R K V D R F N G V S E A (104)  
GAG CTT CTG ACC ACT CTT CCT GAC ATT TTG ACC TTC AAT ATT GTG ATC ATT 480  
E L T T K T L P D I L T F N L D I V I I (124)  
GGC ATT AAC CCG GGA TTA ATG GCT GCT TAC AAA GGA CAT CAC TAC CCT GGG CCT GGA AAT 540  
G I N P G L M A A Y K G H H Y P G P G N (144)  
CAC TTC TGG AAG TGT CTG TTC ATG TCG GGG CTG AGT GAG GTG CAG CTG AAT CAC ATG GAT 600  
H F W K C L F M S G L S E V Q L N H M D (164)  
GAC CAC ACC TTA CCC GGC AAG TAC GGC ATT GGA TTC ACC AAC ATG GTG GAA CGG ACG ACG 660  
D H T L P G K Y G I G F T N M V E R T T (184)  
CCG GGC AGC AAG GAT CTG TCT AGT AAA GAG TTC CGG GAA GGA GGC ATC CTG GTG CAG 720  
P G S K D L S S K E F R E G G R I L V Q (204)  
AAA CTG CAG AAA TAT CAG CCA CGA ATA GCG GTG TTT AAT GGA AAA TGT ATT TAT GAA ATT 780  
K L Q K Y Q P R I A V F N G K C I Y E I (224)  
TTC AGT AAA GAA GTT TTT GGA GTA AAG GTT AAG AAC TTG GAA TTT GGG CTT CAA CCC CAC 840  
F S K E V F G V K N L E F G L Q P H (244)  
AAG ATC CCA GAC ACA GAA ACT CTG TGC TAC GTC ATG CCG TCG TCC AGC GCC AGA TGT GCT 900  
K I P D T E T L C Y V M P S S S A R C A (264)  
CAG TTT CCC CGG GCC CAG GAC AAA GTT CAT TAC TAC ATT AAG CTG AAG GAC TTG AGA GAC 960  
Q F P R A Q D K V H Y Y I K L K D L R D (284)  
CAG CTG AAA GGC ATT GAA CGC AAC ACC GAC GTT CAG GAA GTG CAG TAT ACA TTT GAC CTG 1020  
Q L K G I E R N T D V Q E V Q Y T F D L (304)  
CAG CTT GCG CAA GAG GAC GCA AAG AAG ATG GCT GTT AAG GAA GAA AAG TAT GAT CCA GGC 1080  
Q L A Q E D A K K M A V K E E K Y D P G (324)  
TAT GAG GCA GCT TAC GGC GGC GCC TAT GGG GAA AAC CCA TGT AAT GGG GAA CCT TGT GGC 1140  
Y E A A Y G G A Y G E N P C N G E P C G (344)  
ATT GCT TCA AAT GGG CTA ACA GCC CAC AGT GCG GAG CCG AGA GGA GAA GCG ACC CCC GGC 1200  
I A S N G L T A H S A E P R G E A T P G (364)  
GAT GTT CCA AAT GGG CAG TGG ATG GCA CAG TCG TTT GCA GAG CAG ATC CCT TCT TTT AAT 1260  
D V P N G Q W M A Q S F A E Q I P S F N (384)  
AAT TGT GGG ACC CGA GAG CAG GAA GAA GAG AGC CAC GCT TAG GAA GGG TGC CTC TCA GCT 1320  
N C G T R E Q E E E S H A \* (397)  
CGG GCT CAG GGC TGC AGT TCT AAT GCA GAC CTC AGG ACG GAG AGC CTC AGT GTG CTA ACT 1380  
GAG GGG CTT TAG TGT TAA TGG TCC TTT ACG CTA GTG GTG TAA TTA TAT ATG GAG CAG TTA 1440  
TAT GGT AGA GTC AAC TGT ATG AAT CTG TGT AGT TTG GAA AGG AAA AAT AGG GTG TTT TGT 1500  
ACT TGA GTT TTG TAT TTT AAC TAC CCA TCA TAC CAG CTT CTC ATA TAC TAT ATT TCA TTT 1560  
ATG AAG AAA CTG GTT TTC TCT GAG GAG TCA CTT CAG TCT GTA ATT TTA AAA AAG CAA CAG 1620  
TCG GAA TAT TTA TAG CCT GAT TCT TCA CTG TGC ATA CCT ATA TAT GCC CTT ATC CCT TTT 1680  
AAA TGC CTA AGT AGG GCA TGC TAC TAA GAA TCA AGC TGG TGA AAG CAG ATG TGC CTA TCT 1740  
CTG TTG GAA GCC GAC TAA CCC TCC GCC CTC CGT GGA CTC GAA GCT GGA GGT GGA CTC AGA 1800  
ACC CCT CTG GGT TTG CAG TCA CAC GCA GAC CCC CCT TAC TCT CCC AGT AGT GTG TGT GGC 1860  
AGT TGC TTG GTA GTA GAA ACC TGG TGG GGC CTG AGC CCT GCC GTT GGC CTC TGC CTC TTG 1920  
TGG TTC CTG AGA AGG CAG TAG AAC CTG GAC AAT TGC CAC CCC TCT GTT TTA CTG AAT CCC 1980  
CCG TCA GTG TTC ATC ACT GTT GTG ATT TTA TCC CGA CAT GGA AAG CGC TAA GAA ATG AAC 2040  
TTT CCA GTT AGC CAC GCG CTG CTG GAG GTG TTT GAT AAC CCT GTT TAA ACT GGT GCT GTG 2100  
TAC ATG GGA TGT ACT GAT ACA GAA CTT CTC TTC TAG GGG AAA GCT AAT CAT TTG TCA ATG 2160  
ATT TAA AAA TTA TTG TCA CTA CTG TTG ATT ACT GTG GAC TAA GTG AGC CTC GTG AAA GAG 2220  
CTG ATT GGA ATT TGT ACC TTT GTG GCT GCT CAT GCA TTT TCC ATG GTG CTA CAG ACA GCC 2280  
TGC CCG GGC TGG TGG CTA GGA AAC GTG CGT GTT CAG CGA TGC CTA GCG CTC TCA CTC CTG 2340  
AGC ACT CTG ACT TGG TGC CCA CCC CTG GCG CGT GCT GGT TTG GCA GTA TGA GAT GCC CAC 2400  
GAG GGA TTT GTA TCT CAC GAA GGT TAT GTA GCC TGT TCT GTG AAT AAT GAG GGA AGC AGT 2460  
TTC TTG GTG GAG ATG TTA ACA TAC CTT TAG CTG TGG CAG TGA TGG GCT TTC TTT TTC CTT 2520  
CTG TCT GGG TCA GGT TGT GCA CAA GTC ATG TTA GCT GGA AGG TGC ACT GCA CTG CTG TTT 2580  
ACA TGG GCA TTT TAT GCG GGT GCA TTG AAG TGC CTT GCA TCA GCG ATT AGT GTC AAG TGA 2640  
ACT CTT TGT CAT GGG ACT ATG TCA AGC ATG TAG CAT TGC CGT GGT ATG TAA CTA TTG TAG 2700  
TTT TGC CAT GGA GAA ATA CCT TTA AAT TAT AAC TCT GGT GTC CCG TGA GGA CCC TCT 2760  
GTA CTT CCT GGT GCT TTT CCT TGA CAT GGT GGG TGA GTA ACG AGT CCG GGG CAC CCT GTA 2820  
CAT GGC AGG GTG GGA CTT TCT CAT TAA ATA ATG CTG AGA 2859

## B

mTDGa	1	MDAEARSYS	LEQVQALYSF	PFOQMAEVP	NMAVTITGQOV	PAVAPNMATV	TEQQVPADAP	VOEPAPEAPK	RRKRKPRAAE	POEPVEPKKP	ATSKKSGKST	100
mTDGb	1	.....	.....	...MMAEVP	NMAVTITGQOV	PAVAPNMATV	TEQQVPADAP	VOEPAPEAPK	RRKRKPRAAE	POEPVEPKKP	ATSKKSGKST	76
hTDGa	1	MEAEINAGSYS	LQQAQAFYTF	PFOQLMAEAP	NMAVVNEQQM	P.....	..EEVPAPAP	AQEPVQEAAPK	GRKRKPRTTE	PQQVPEPKKP	VESKKSCKSA	89
mTDGa		KSKEKQEKIT	DAFKVKRKVD	RFNGVSEAEI	LTKTLDPILT	FNLDIVIIGI	NPGLMAAYKG	HHYPGPGNHF	WKCLFMSGLS	EVQLNHHMDH	TLPGKYGIGF	200
mTDGb		KSKEKQEKIT	DAFKVKRKVD	RFNGVSEAEI	LTKTLDPILT	FNLDIVIIGI	NPGLMAAYKG	HHYPGPGNHF	WKCLFMSGLS	EVQLNHHMDH	TLPGKYGIGF	176
hTDGa		KPKKEKQEKIT	DTFVKRKVD	RFNGVSEAEI	LTKTLDPILT	FNLDIVIIGI	NPGLMAAYKG	HHYPGPGNHF	WKCLFMSGLS	EVQLNHHMDH	TLPGKYGIGF	189
mTDGa		TNMVERTTPG	SKDLSSEKFR	BGGRILVQKL	QKYQPRIAVF	NGKCIYEIFS	KEVFGVKVKN	LEFGLQPHKI	PDTETLCYVM	PSSSARCAQF	PRAQDKVHY	300
mTDGb		TNMVERTTPG	SKDLSSEKFR	BGGRILVQKL	QKYQPRIAVF	NGKCIYEIFS	KEVFGVKVKN	LEFGLQPHKI	PDTETLCYVM	PSSSARCAQF	PRAQDKVHY	276
hTDGa		TNMVERTTPG	SKDLSSEKFR	BGGRILVQKL	QKYQPRIAVF	NGKCIYEIFS	KEVFGVKVKN	LEFGLQPHKI	PDTETLCYVM	PSSSARCAQF	PRAQDKVHY	289
mTDGa		IKLKDLRDQL	KGIERNTDVQ	EVQYTFDLQL	AQEDAKKMAV	KEEKYDPGYE	AAYGGAYGEN	PCNGEPCGIA	SNGLTAHSAA	PRGEATPGDV	PNGQWMAQSF	400
mTDGb		IKLKDLRDQL	KGIERNTDVQ	EVQYTFDLQL	AQEDAKKMAV	KEEKYDPGYE	AAYGGAYGEN	PCNGEPCGIA	SNGLTAHSAA	PRGEATPGDV	PNGQWMAQSF	376
hTDGa		IKLKDLRDQL	KGIERNMDVQ	EVQYTFDLQL	AQEDAKKMAV	KEEKYDPGYE	AAYGGAYGEN	PCSSEPCGFS	SNGLI.EEVE	LRGESAFSGI	PNGQWMTQSF	388
mTDGa		AEQIPSPFN.N	CGTREQEES	HA	421							
mTDGb		AEQIPSPFN.N	CGTREQEES	HA	397							
hTDGa		TDQIPSPFNH	CGTREQEES	HA	410							



primers (see "Experimental Procedures") on poly(A) mRNA from P19 embryonal carcinoma cells (data not shown). However, further Northern blot analysis using poly(A) mRNAs from various mouse adult tissues and a cDNA probe encoding a region common to the two isoforms (bp 286–2567 of TDGb cDNA) revealed only one mRNA species of ~3 kilobases (Fig. 2A) that was present in all tissues tested.

A monoclonal anti-TDG antibody was used to analyze TDG protein levels in various monkey, mouse, and human cell lines (Fig. 2B). TDG was detected in all tested cell lines with the lowest amount in F9 teratocarcinoma cells. In all extracts, two protein species of ~63 kDa were revealed which comigrated with *E. coli*-expressed mTDGa(32–421), thus suggesting that the two species could represent TDGa and TDGb isoforms (see below). The mouse TDG was slightly larger than monkey and human TDG. In addition to the ~63-kDa species, the antibody revealed in some extracts two slower migrating forms of the protein with an apparent molecular mass of ~90 kDa. The relative level of these species varied in different extract preparations from the same cell line, suggesting that they may represented modified forms of the enzyme. As TDG contains a number of putative kinase sites, MCF7 cell extracts were treated with CIP in the presence and absence of vanadate as a phosphatase inhibitor. In the absence of the phosphatase inhibitor, CIP converted the majority of the upper species into forms migrating with an apparent molecular mass of ~63 kDa (Fig. 2C).

To further characterize the two TDG species of ~63 kDa, the mouse TDGa cDNA was cloned by PCR and expressed in transfected COS cells along with mTDGb cDNA. Extracts from transfected COS cells were analyzed by Western blotting together with an extract from P19 cells. Overexpressed mTDGa comigrated in this experiment with the upper species from P19 cells whereas mTDGb migrated at the level of the lower species (Fig. 2D). Similar comigrations were also observed for the larger protein species recognized by the TDG antibody. Since P19 cells were shown to contain transcripts for the TDGa and TDGb isoforms (see above), it is likely that the two TDG protein species found in extracts of various cells correspond to TDG isoforms a and b. In all cell lines and tissues analyzed by Western blotting, these two TDG isoforms were found in similar amounts (Fig. 2B and data not shown).

**TDG Interacts with RAR and RXR in Yeast and in Vitro**—Full-length mTDGa was fused to the VP16 acidic activation domain (denoted AAD) and assayed for interaction with full-length RAR $\alpha$  and RXR $\alpha$  fused to LexA in the yeast strain L40 which contains a *lacZ* reporter gene driven by eight LexA binding sites (49). Interestingly, while a very weak (~5-fold), but reproducible, increase in  $\beta$ -galactosidase activity was obtained by co-expressing AAD-TDGa with unfused LexA, 335- and 75-fold enhancements were observed by co-expressing AAD-TDGa with LexA-RXR $\alpha$  and LexA-RAR $\alpha$ , respectively, in the absence of ligand (Fig. 3A). Addition of 9c-RA to yeast expressing LexA-RXR $\alpha$  and AAD-TDGa or t-RA to yeast expressing LexA-RAR $\alpha$  and AAD-TDGa diminished by a factor of ~2 and 3, respectively, the -fold enhancement mediated by AAD-TDGa over the AAD control (Fig. 3A), indicating that TDGa may interact preferentially with the unliganded forms of RXR $\alpha$  and RAR $\alpha$  in yeast. As a representative of the other conserved gene family of DNA glycosylases (see Introduction), the human uracil-specific U:G mismatch-specific DNA glycosylase (hUNG1) (58, 59) was tested for its ability to interact with

RXR $\alpha$  and RAR $\alpha$  in yeast. Similarly to AAD-TDGa, co-expression of AAD-UNG1 with unfused LexA resulted in a weak activation of the reporter gene (Fig. 3A). However, no further stimulation was observed by co-expressing AAD-UNG1 with LexA-RXR $\alpha$  or LexA-RAR $\alpha$  in either the presence or absence of ligand (Fig. 3A), indicating that, in contrast to TDGa, UNG1 does not interact with RXR $\alpha$  and RAR $\alpha$ .

TDG was then tested for a direct binding to RAR and RXR *in vitro*. GST pull-down assays using Vaccinia virus-expressed TDGa(32–421) (Fig. 3B) or *in vitro* translated full-length TDGa (data not shown) and *E. coli*-expressed GST-RAR $\alpha$  or RXR $\alpha$  fusion proteins revealed a ligand-independent interaction between TDG and the receptors.

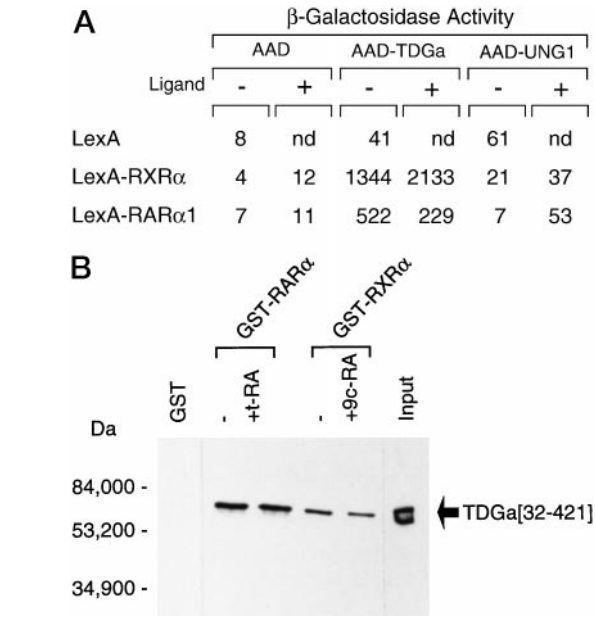
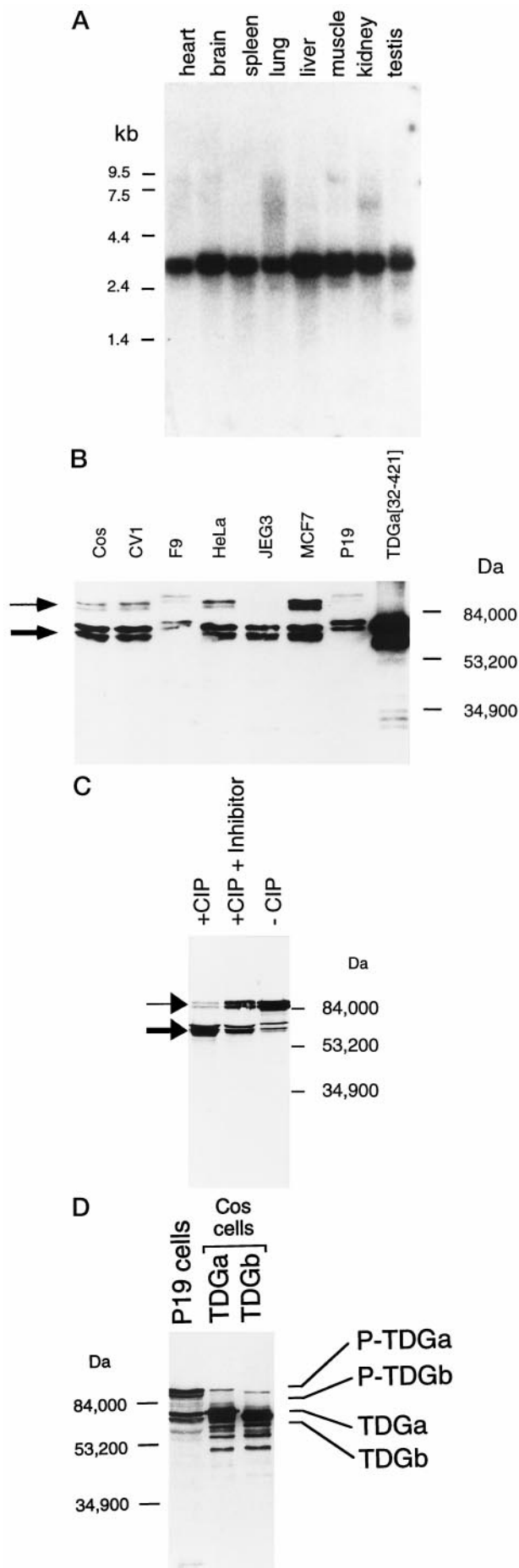
To map the interaction domains of the nuclear receptors and TDG, a deletion analysis of each protein was performed using the yeast two-hybrid system. Deletion derivatives of RXR $\alpha$  were expressed as fusion proteins with LexA and tested for interaction with AAD-TDGa(32–421) (Fig. 4A). Similarly to AAD-TDGa, AAD-TDGa(32–421) interacted with RXR $\alpha$  in both the presence and absence of 9c-RA (Fig. 3A and 4A). A region between residues 227 and 263 harboring the  $\alpha$ -helix 1 (H1) of the LBD of RXR $\alpha$  was found to be sufficient for mediating an interaction with TDG (Fig. 4A, see *LexA-RXR $\alpha$ [227–263]* and *AAD-TDG[32–421]*). Interaction with the whole LBD of RXR $\alpha$  (amino acids 206–467) exhibited a 3-fold increase in the presence of 9c-RA (Fig. 4A). In view of this ligand-dependent enhancement, the effects of point mutations in the core motif of the AF-2 activation domain (AF-2 AD core/helix 12 of the LBD) were studied. Out of the three mutations tested (FL to AA for aa 455 and 456, ML to AA for aa 459 and 460, and E to Q for aa 461), only amino acid changes in positions 455 and 456 reduced significantly the interaction with TDG (Fig. 4A). This indicates that not all of the conserved residues of the AF-2 AD core, which are important for transactivation by the ligand-dependent AF-2 (11), are required for the binding of TDG to the LBD of RXR $\alpha$ .

Similarly, deletion derivatives of RAR $\alpha$  were examined to localize the region(s) required for interaction with TDG in yeast. No significant increase in reporter activity was observed with the N-terminal A/B region of RAR $\alpha$  (Fig. 4B; *LexA-RAR $\alpha$ [1–88]*). In contrast, a ~170-fold activation was detected in the presence of the LexA-RAR $\alpha$ (80–211) fusion protein containing the DNA-binding domain (region C) and D region of the receptor (Fig. 4, B and C). Co-expression of a LexA fusion containing the C region only (LexA-RAR $\alpha$ (80–155) with AAD-TDG(32–421) resulted in a modest (~10-fold) activation of the reporter gene (Fig. 4B), whereas a C-terminal fragment of the D region encompassing the  $\alpha$ -helix 1 of the LBD (amino acids 170–211) interacted strongly with TDG (Fig. 4B). Thus, as observed with RXR $\alpha$ , the helix 1 of the LBD of RAR $\alpha$  is sufficient for mediating an interaction with TDG. However, in contrast to the RXR $\alpha$  LBD, the whole LBD of RAR $\alpha$  (amino acids 154–462) showed no ligand-dependent interaction with TDG (Fig. 4B).

To map the receptor interacting domain in TDG, various deletion mutants of TDG were fused to the VP16 AAD and assayed for interaction with LexA-RXR $\alpha$ [DE] in yeast. These experiments revealed a central region of TDG between amino acids 122 and 346 as sufficient for the interaction with RXR $\alpha$  (Fig. 4C) and RAR $\alpha$  (data not shown). Further deletions in that region abolished interactions with the receptors.

Since TDG was initially cloned as a c-Jun interacting protein

FIG. 1. **Cloning of mouse TDG isoforms.** A, nucleotide and amino acid sequences of the 2859-bp mTDGb cDNA clone. The first ATG codon is underlined. The deduced amino acid sequence in front of the first methionine is shown in lowercase. B, alignment of mouse TDGa (mTDGa; (39)), mouse TDGb (mTDGb; this study), and human TDGa (hTDGa; (39)). Dots represents gaps used to align the sequences.

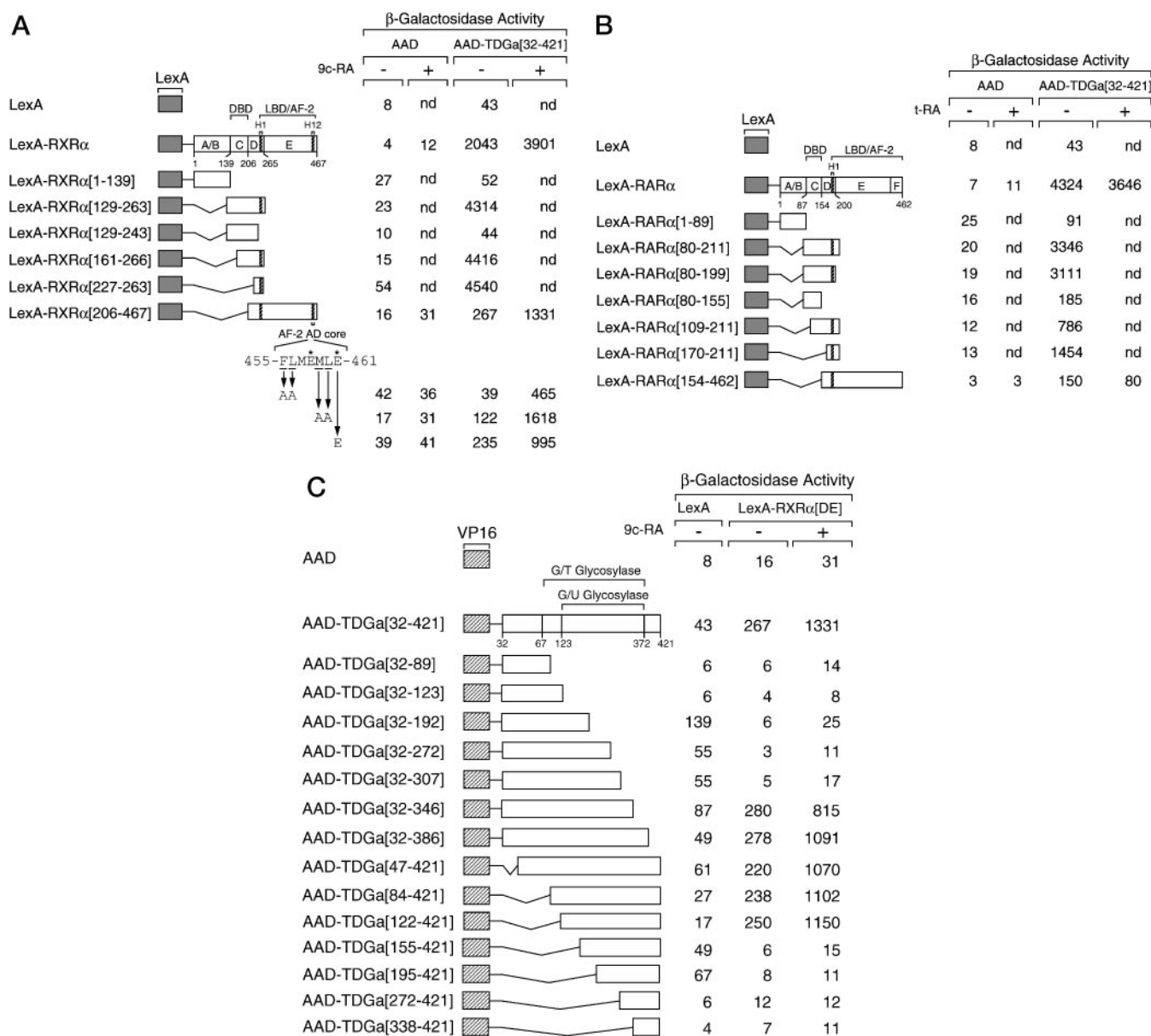


**FIG. 3. TDG interaction with RXR and RAR.** *A*, TDG, but not UNG1, interacts with RXR and RAR in a yeast two-hybrid system. Plasmids expressing either “unfused” LexA or LexA fused to mRXRα or hRARα1 were co-transformed in the yeast reporter strain L40 with either the VP16 AAD or the VP16 AAD linked to mTDGa or hUNG1. Transformants were grown in liquid medium in the presence or absence of 500 nM ligand (9c-RA for RXR and t-RA for RAR) for about five generation times. β-Galactosidase activities determined on each cell-free extracts were expressed in nanomoles of substrate/min/mg of protein. The values (± 20%) are the averages of at least three independent transformants. *B*, TDG interacts with RXR and RAR *in vitro*. About 5 μg of GST-mRXRα or GST-hRARα1 were bound to a glutathione-Sepharose matrix and incubated with 100 ng of Vaccinia virus expressed mTDGa(32–421) in the presence or absence of 1 μM 9c-RA (for RXR) or t-RA (for RAR). Proteins bound to the beads were eluted and analyzed by Western blotting.

(42), we used the yeast two-hybrid assay to investigate the interaction of the C-terminal part of c-Jun (amino acids 247–331) with the different TDGa deletions. The same central region of TDG from position 122 to 346 was found to be required for the binding to c-Jun (data not shown).

**TDG Enhanced Transactivation by RXR and RXR/RAR**—To investigate whether the interactions between TDG and either RAR or RXR could affect the transactivation mediated by RXR homodimers or RXR/RAR heterodimers, transient transfection experiments with increasing amounts of TDGa(32–421) were performed in COS cells. Ligand-induced transactivation by RXRα from a single DR1 element in a DR1(G)-tk-CAT reporter gene enhanced up to 4-fold by addition of TDGa(32–421) (Fig.

**FIG. 2. Expression of mTDG in various tissues and cell lines.** *A*, an approximately 3-kilobase RNA transcript is revealed in various mouse tissues by Northern blot analyses. *B*, Western blot detection of two TDG species with an apparent molecular mass of ~63 kDa (arrow) of whole cell extracts from COS, CV1, F9, HeLa, JEG3, MCF7, and P19 cells as indicated. In addition, larger forms of TDG were detected with an apparent molecular mass of ~90 kDa (arrow). In the last lane, 25 ng of *E. coli*-expressed mTDGa(32–421) were loaded as a control. *C*, dephosphorylation of TDG. 25 μg of a whole cell extract from MCF7 cells were incubated for 30 min with 20 units of CIP in the presence or absence of 50 μM vanadate (inhibitor). After the reaction the different forms of TDG were revealed by Western blotting. *D*, TDGa and TDGb are present in P19 cells. COS cells were transfected with 1 μg of pSG5-mTDGa or pSG5-mTDGb, and whole cell extracts were prepared 24 h after transfection. These extracts from transfected COS cells and a whole cell extract from mouse P19 cells were analyzed by Western blotting. The two upper species, P-TDGa and P-TDGb, correspond to the phosphorylated forms.



**FIG. 4. Mapping of the TDG-nuclear receptor interaction domains.** A, residues 227–263 of the RXRα LBD harboring helix 1 (H1) are sufficient for interaction with TDG, and integrity of the AF-2 AD core (helix 12/H12) is not required for the ligand-dependent interaction of the RXRα LBD with TDG. Plasmids expressing RXRα or deletion mutants of RXRα fused to LexA were cotransformed into yeast L40 with AAD or AAD-TDGa(32–421). Transformants were grown in the presence or absence of 500 nM 9c-RA. β-Galactosidase activity is expressed as in Fig. 3A. B, residues 170–211 of the RARα LBD harboring helix 1 (H1) are sufficient for interaction with TDG. L40 transformants expressing the indicated LexA and AAD fusion proteins were grown in the presence or absence of 500 nM t-RA. β-Galactosidase activity is expressed as in Fig. 3A. C, residues 122–346 of TDG are required for interaction with the LBD of RXRα. Various deletions of TDG were fused to the VP16 AAD and co-expressed with LexA or LexA-RXRα(264–467) in the yeast L40 strain grown in the presence or absence of 500 nM 9c-RA. β-Galactosidase activity is expressed as in Fig. 3A. In panels A–C, all fusion proteins were expressed in yeast as assayed by Western blotting (data not shown).

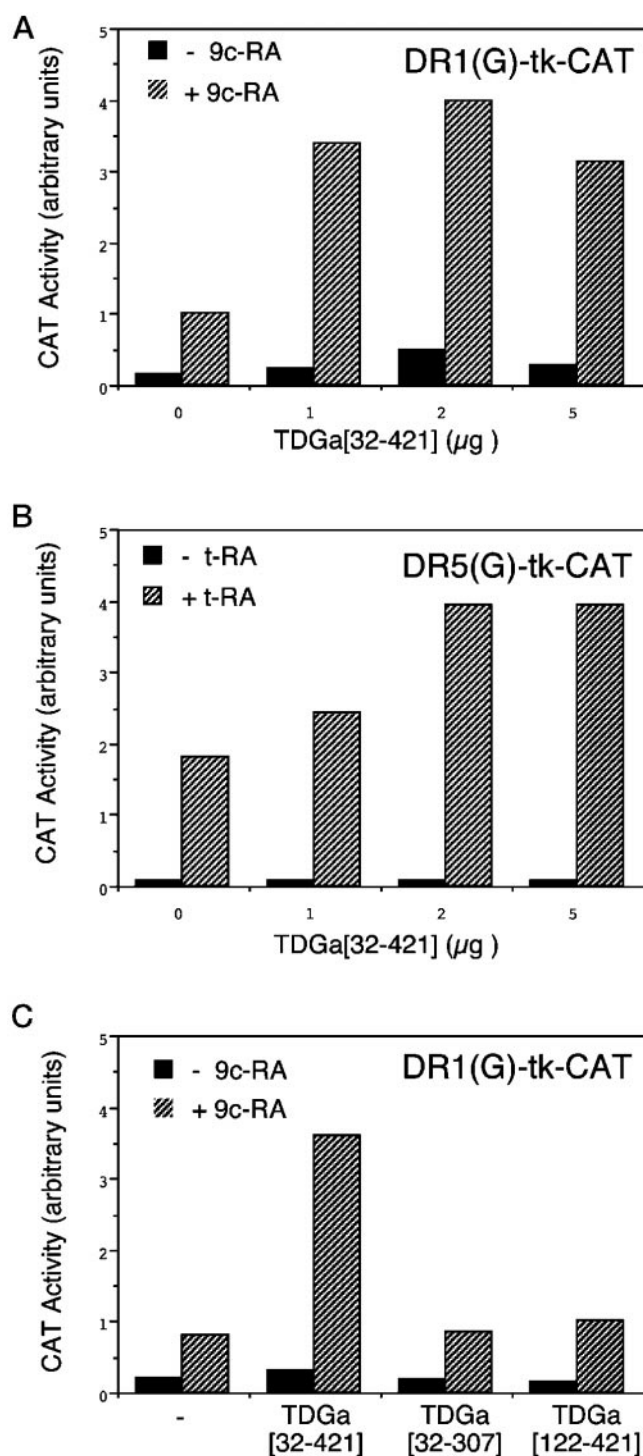
5A). In similar transfection assays using a DR5(G)-tk-CAT reporter gene cotransfected with RARα and RXRα expression vectors, t-RA induced activation from one DR5 element increased by 2–3-fold (Fig. 5B). Control experiments using a reporter gene construct lacking a RARE indicated that overexpression of TDG had no effect on transcription from the tk promoter (data not shown). The truncated TDGa(32–307), which could not bind to RXRα (see Fig. 4C), did not enhance transactivation from a DR1(G)-tk-CAT reporter gene (Fig. 5C). Similarly, the N-terminally truncated mutant TDGa(122–421) was unable to increase transactivation by RXRα, although it could still interact with RXR in yeast (see Figs. 4C and 5C). This observation points to a requirement of the N terminus of TDG for enhancement of receptor-mediated transcription. However, experiments with various TDG fusion proteins in

yeast and mammalian cells did not reveal any transactivation domain in the N terminus or any other part of TDG (data not shown).

As TDG is known to interact also with c-Jun (Ref. 42 and see above), we investigated in transient transfection assays whether it could be involved in transrepression between the retinoic acid receptors and AP1 (62–64). However, overexpression of TDGa(32–421) in COS and HeLa cells did not relieve the transrepression exerted by RAR/RXR on the activation of the stromelysin or collagenase promoter by AP1, nor the transrepression exerted by AP1 on RXR/RAR-mediated transactivation from promoter regions containing RAREs (data not shown).

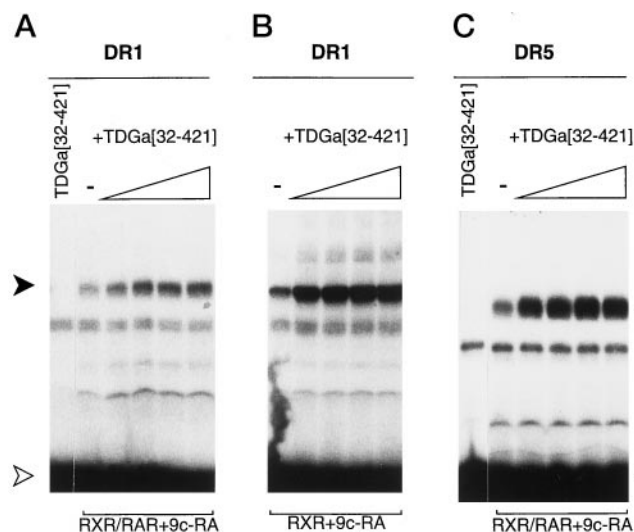
*TDG Enhanced the Binding of RXR and RXR/RAR to DNA in Vitro*—EMSA were performed to investigate whether TDG





**FIG. 5. TDG enhances transactivation by RXR and RXR/RAR.** A, TDG stimulates the 9c-RA-induced transactivation by RXR. COS cells were transfected with 1 μg of DR1(G)-tk-CAT reporter gene along with 1 μg of pSG5-mRXRα and increasing amounts of pSG5-mTDGa(32–421) as indicated. Twelve hours after transfection, 500 nM 9c-RA was added for 24 h when indicated before measuring CAT activity in whole cell extracts. All values were standardized for β-galactosidase activity. B, TDG stimulates the t-RA-induced transactivation by RXR/RAR. COS cells were transfected with 1 μg of DR5(G)-tk-CAT reporter gene plasmid, 1 μg of each pSG5-mRXRα and pSG5-hRARα and treated for 24 h with 500 nM t-RA when indicated. C, effect of TDG deletions on the RXR-mediated transactivation. Transfection experiment was performed as in A with 2 μg of mTDGa(32–421), mTDGa(32–307), or mTDGa(122–421).

could enhance the binding of RXR or RXR/RAR to their response elements. Liganded RXRαΔAB was studied for its binding to a DR1 element (Fig. 6B) as well as liganded RXRαΔAB/



**FIG. 6. TDG increases DNA binding by RXR homodimers and RXR/RAR heterodimers *in vitro*.** For electrophoresis mobility shift assays, 50 ng of purified RXRΔAB or RXRΔAB/RARΔAB heterodimer were incubated with increasing amounts of Vaccinia virus expressed mTDGa(32–421) (50, 100, 150, or 200 ng) and 5 ng of <sup>32</sup>P-labeled oligonucleotides representing a DR1 or DR5 binding site. Protein-DNA complexes (closed arrowheads) and free oligonucleotides (open arrowheads) were separated on a 6% polyacrylamide gel. A, RXR/RAR binding to a DR1 element; B, RXR binding to a DR1 element; C, RXR/RAR binding to a DR5 element.

RARαΔAB heterodimers for their binding to DR1 and DR5 elements in the presence of increasing amounts of Vaccinia virus-expressed TDGa(32–421) (Fig. 6, A and C). Although no TDG-receptor complex could be identified in these experiments, in all cases the addition of TDGa(32–421) led to an approximately 3-fold increase in DNA-binding by RXRαΔAB and RXRαΔAB/RARαΔAB. Under similar EMSA conditions, TDGa(32–421) could not bind to DR1 or DR5 response elements on its own.

#### DISCUSSION

In a screening for factors supporting RAR to function as a ligand-dependent transcription factor in yeast, we have identified TDG as a protein that can interact with RAR and RXR in yeast as well as *in vitro*. In a yeast two-hybrid screening, TDG was previously cloned as a c-Jun interacting protein (42), and later identified as a T:G and U:G mismatch-specific DNA repair enzyme (39).

A new TDG isoform-specific cDNA (called b) has been cloned, which differs in the 5' sequences from the previously published mouse and human cDNAs (denoted isoform a) (39). These cDNAs are likely to correspond to alternative splicing variants of the same transcript. Although Northern blots analysis detected only one RNA species in all mouse tissues tested, transcripts corresponding to the TDG isoforms a and b could be detected in P19 embryonal carcinoma cells. Using monoclonal anti-TDG antibodies, two major TDG species were revealed in whole cell extracts from various mouse, monkey, and human cell lines as well as mouse tissues. These two forms of TDG are probably encoded by the two different transcripts found in P19 cells. Alternatively, these two species could be translated from TDGa mRNA using two different starting methionines as discussed for the human enzyme (39). However, the cloning of additional splice variants from mouse suggests that similar transcripts could also exist in humans. As the first 25 amino acids of the TDGa isoform, which are lacking in TDGb, are dispensable for both the binding to RAR or RXR and the enzymatic activity (40), the functional significance of the two isoforms is unknown. In addition to the two TDGa and TDGb



isoforms, Western blot analyses and phosphatase treatment of cell extracts indicated the existence of phosphorylated forms of the TDG isoforms. The significance of this modification is also unknown, although it does not appear to be required for *in vitro* interactions between TDG and RAR or RXR.

In the present study, we have shown that TDG can interact with RAR and RXR, both in yeast and *in vitro* in a ligand-independent manner. Mapping of the binding sites revealed interaction with a region in the RAR and RXR LBD harboring the  $\alpha$ -helix 1 and a central region of TDG between amino acids 122 and 346. An alignment of hTDG with the related U:G-specific DNA glycosylase from *E. coli* (MUG) has shown that the TDG region from amino acid 122 to 346 is the most conserved one and that it also contains the enzymatic center of the glycosylase (40). Interestingly, the same region of TDG appears to be required for its binding to c-Jun and its homodimerization in yeast,<sup>3</sup> suggesting that it could participate in various protein-protein interactions.

Transfection of TDG in COS cells increased RXR- and RXR/RAR-mediated transactivation from reporter genes containing cognate response elements for the receptors. The observation that the truncated TDG(32–307) could not bind to the receptors and failed also to enhance RXR-induced transcription suggests that the interaction between TDG and the receptors could be of functional importance. To investigate the basis for the increased transactivation by RXR and RAR in the presence of TDG in transient transfection experiments, TDG was fused to various DNA-binding domains as those of the yeast transcription factor GAL4, the estrogen receptor, or the *E. coli* regulator LexA. Using these fusion proteins in yeast or in transfected COS cells with cognate reporter genes did not reveal any transcriptional activation domain in TDG (data not shown). However, TDG was shown to enhance the DNA binding of either RXR homodimers or RXR/RAR heterodimers to cognate DR elements, arguing that TDG can enhance RXR- and RXR/RAR-mediated transactivation by stabilizing their binding to DNA. In this context, it is interesting to note that the N-terminal part of TDG (amino acids 70–122) is rich in basic amino acids sharing in part similarity with a HMG box motif (HMG-I/HMG-Y family). This TDG region is required for its T:G-specific, but not for its U:G-specific DNA glycosylase activity, suggesting that it contributes to the enzyme selectivity by contacting DNA (40). Interestingly, HMG-1 was found to stabilize binding of the progesterone receptor to its target sequences in EMSA (66, 67) and to enhance transactivation by estrogen receptor at the level of DNA binding (68). In the case of the progesterone receptor, a model was proposed in which HMG-1 first bends the DNA, and dissociates from DNA after formation of a stable receptor-DNA complex (66). The N-terminal region of TDG may act similarly to support receptor-DNA binding. Accordingly, a truncated mTDG(122–421) lacking the basic region could not enhance the RXR-mediated transactivation in transient transfection assays, even though it could still interact with the receptors. During the last years, an increasing number of putative coactivators was cloned which have been characterized for their binding to nuclear receptors (5, 6). TDG is the first to be shown to act at the level of DNA binding. Interestingly, TDG can also interact with the putative nuclear receptor cofactor TIF1 $\alpha$ .<sup>3</sup> This interaction may generate additional possibilities through which TDG could affect the transcriptional activity of nuclear receptors.

The effect of TDG on RXR- and RXR/RAR-dependent transcription was specific to nuclear receptors, since TDG expression had no effect on the transcriptional activity of GAL4 DNA-

binding domain fusion proteins containing the activation domains of SP1, AP2, Oct1, or VP16 (data not shown). However, the transcriptional activity of a GAL4-cJun fusion protein was increased by overexpression of TDG.<sup>4</sup> This enhancement may involve the previously reported interaction between c-Jun and TDG (42). As a component of the AP1 transcription factor c-Jun can participate in transrepression of RAR and RXR (63). As TDG can interact with RAR and RXR as well as with c-Jun, we investigated whether TDG could be involved in transrepression. TDG overexpression had no significant effect on transrepression of RXR and RAR by AP1 or on repression of AP1 by RAR and/or RXR.<sup>5</sup> Thus, transrepression between AP1 and the receptors does not appear to be caused by a competition for limiting amounts of TDG.

DNA repair and transcription are linked by a number of factors participating in both processes (69) such as the c-Jun and p53 activating A/P endonuclease Ref-1 (70, 71) or the basic transcription/repair factor TFIIF (72). The fact that TDG can interact with different transcription factors like c-Jun, RAR, RXR, or other nuclear receptors and a transcription-related protein like TIF1 $\alpha$  suggests that TDG could be another DNA repair enzyme which functions also in transcription. The use of DNA repair factors like TDG in transcription could therefore be one mechanisms of how to maintain the integrity of transcribed regions.

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<sup>4</sup> S. Um, unpublished data.

<sup>5</sup> S. Um, M. Harbers, A. Benecke, B. Pierrat, R. Losson, and P. Chambon, unpublished data.

<sup>3</sup> M. Harbers, unpublished data.

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