Discussion

The present paper documents a significant increase in the survival of crypts in animals receiving 5% MAK associated with a prolongation of average time to animal death after X-irradiation. Hsu *et al* (4,19) earlier reported that intraperioneal injections of the extract from *Ganoderma lucidum* before irradiation of 5 or 6.5 Gy X-rays improved the 30-day survival of ICR mice and increased recovery as assessed by hemograms, the 10-day blood forming stem cells (CFU) also being significantly higher for the *Ganoderma lucidum* treated group than for the untreated group. Chen *et al* (20,21) reported that administration of an extract of *Ganoderma lucidum* was able to enhance the recovery of cellular immunocompetence after 4 Gy-ray irradiation of ICR mice. It is well documented that radiation is a potent immunosuppressive agent, and moderate doses exert clear inhibitory effects on the counts of total leukocytes, lymphocytes and neutrophils. Radiation also has destructive effects on the leukocytepoietic organs such as the spleen, thymus and bone marrow, and protection effects of traditional Chinese medicines (1-4,19), ginseng (12,22,23) and garlic (24) have been investigated. They were able to enhance the recovery from decreased cellular immunocompetence, with protection or stimulation of the reticuloendothelial system, and induction of free radical scavenger. Recently, we reported that a water-soluble extract from culture medium of *Ganoderma lucidum* mycelia (designed as MAK) may stimulate the natural immune system or the aquire immune system in tumor-bearing mice (25). Therefore MAK might be a potent immunomodulater that up-regulates against immunosuppression by X-irradiation.

Houchen *et al* (26) have reported that expression of FGF-2 is induced by radiation injury and that recombinant human FGF-2 markedly enhanced crypt survival. Takahama *et al* (27) found that a replication-deficient adenovirus containing the HST-1 gene acts as a potent protector against lethal irradiation associated with injury to the intestinal tract as well as myelosuppression in the bone marrow and spleen. Farrell *et al* (28) have presented findings that recombinant human keratinocyte growth factor can protect mice from chemotherapy- and radiation-induced gastrointestinal injury and mortality, at least in terms of death from intestinal and marrow toxicity. We also found VEGF to have a protective influence (29). Cytokine-like substances in MAK may thus play an important role in the protection and/or the recovery and repopulation of critical tissue elements when given prior to and during radiation exposure. However, to our knowledge, there are no reports regarding cytokines in MAK.

MAK contains various kinds of high molecular constituents, i.e. polysaccharides with protein or water-soluble lignin, and low molecular constituents, i.e. triterpenes. *Ganoderma lucidum* mycelia were cultured in a solid medium composed mainly of sugar-cane bagasse for 3 months. Lignin is processed or converted to water-soluble lignin with enzymes during growth of mycelia. A water-soluble lignin is seemed to be the characteristic constituent, and does not contribute to every function of MAK, but is closely related to some function of MAK.

The present experiment provided evidence of recovery of seminiferous tubule size and DNA synthesis with MAK, but not Agaricus treatment. The testis is the most sensitive organ for radiation injury (30,31) and the turnover time from primary spermatogonia to sperm is 60 days (32). Thus, both the smaller testis size and number of PCNA-positive cells of seminiferous tubes in the Agaricus group as compared with the MAK group, are compelling evidence of less radiation protection.

Studies are now in progress in our laboratory to further elucidate the active compounds of the water-soluble extract, the mode of their action included immune parameter and further clinical study for pharmaceutical effects.

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The Human Pre-B Cell Line Nalm-6 Is Highly Proficient in Gene Targeting by Homologous Recombination

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ABSTRACT

Gene targeting provides a powerful means for analyzing gene function, as exemplified by knockout mouse studies and recent work with the highly recombinogenic chicken DT40 B-lymphocyte line. In human cultured cells, however, the low frequency of gene targeting is a serious barrier to efficiently generate knockout clones. Moreover, commonly used human cell lines are karyotypically abnormal or unstable. Here, we show using promoterless targeting constructs that Nalm-6, a human pre-B ALL cell line, is highly proficient for gene targeting by homologous recombination. Indeed, the efficiency of *TP53* gene targeting in Nalm-6 appears nearly two orders of magnitude higher than that in HCT116, a colon cancer cell line popularly used for gene targeting. Expression analysis revealed a lack of *MSH2* expression in this cell line. As Nalm-6 has a stable near-diploid karyotype with normal p53 status, our results underscore the usefulness of Nalm-6 for gene knockout studies in humans.

INTRODUCTION

GENE TARGETING provides a powerful means for studying gene function by a reverse genetic approach (Capecchi, 1989; Vasquez *et al.*, 2001). This technology relies on a homologous recombination reaction that occurs, albeit rarely, between transfected DNA (i.e., targeting construct) and the host genome. In mice, a number of genes have been knocked out so far using embryonic stem (ES) cells, and their physiological functions have been elucidated. More recently, reverse genetic studies with the chicken B-lymphocyte DT40 cell line have made a significant contribution to our understanding of cellular functions of various genes, especially those involved in DNA repair and recombination (Yamazoe *et al.*, 2004).

It should be kept in mind, however, that the findings in rodent or avian cells are not always the case in human cells because of species difference and distinct genetic backgrounds. Moreover, recent evidence suggests that despite extensive sequence similarities, some rodent or avian genes can be functionally different from their human counterparts (Li *et al.*, 2002). Therefore, reverse genetic analysis using human somatic cells would be of greater importance in the postgenome era when we attempt to reliably analyze the function of human genes, particularly toward diagnostic and therapeutic applications. In human cultured cells, however, the frequency of gene targeting is typically too low for such analysis to be feasible (Yanez and Porter, 1998; Vasquez *et al.*, 2001). Moreover, commonly used human cell lines are, unlike ES cells, cancer-derived, and thus karyotypically abnormal or unstable. Accordingly, gene-targeting experiments of human genes have only been performed in a limited number of cell lines. Among these cell lines, popularly used is the HCT116 colon cancer cell line (Bunz *et al.*, 1998) and "promoterless" constructs (where a positive selection marker has no promoter sequence) are preferentially used to maximize the efficiency of gene targeting, which yet is considerably low in HCT116 cells relative to mouse ES or avian DT40 cells.

Recently, Lieber and coworkers have reported high gene-targeting efficiencies in the human pre-B cell line Nalm-6 with the use of targeting constructs for the *LIG4* gene (Grawunder *et al.*, 1998). This finding prompted us to knock out the *BLM* gene (responsible for Bloom's syndrome) in Nalm-6 cells, which has indeed been successfully done with similarly high gene-targeting efficiencies (So *et al.*, 2004). Importantly, tar-

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geting constructs used in those studies were not "promoterless"; instead, a negative selection marker (i.e., a gene encoding diphtheria toxin A fragment) was added to each construct to select against unwanted random integrants, as has been conventionally used for mouse ES cell gene targeting. Thus, the effectiveness of promoterless constructs has yet to be evaluated in Nalm-6 cells.

In this report, we use promoterless constructs to perform targeted gene disruption of in Nalm-6 cells. We demonstrate that Nalm-6 is in fact highly proficient for gene targeting, as evidenced by significantly higher gene-targeting efficiencies at the *TP53* locus than those achieved in HCT116 cells. The usefulness of Nalm-6 in gene targeting led us to perform genome-wide expression analyses, which revealed a lack of expression of MSH2, a protein that plays a critical role in DNA mismatch repair (Buermeyer *et al.*, 1999; Stojic *et al.*, 2004). Our data provide valuable information for gene-knockout studies with the Nalm-6 cell line.

MATERIALS AND METHODS

Cell culture and transfection

Human Nalm-6 cells were cultured in ES medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 10% calf serum (Hyclone Labs, Logan, UT) and 50 μM 2-mercaptoethanol at 37°C in a humidified atmosphere of 5% CO_2 in air. Nalm-6 derivatives lacking BLM (So *et al.*, 2004) or DNA ligase IV (our unpublished results) have been created by targeted gene disruption. DNA transfection was performed as described previously (Adachi *et al.*, 2001). Briefly, 4×10^6 cells were electroporated with 4 μg of targeting construct, and incubated at 37°C for 2–3 weeks in agarose medium containing either 0.2–0.4 mg/ml hygromycin B (Wako Pure Chemical, Osaka, Japan) or 0.5–1.5 mg/ml G418 (Sigma-Aldrich, St. Louis, MO). Genomic DNA was isolated from drug-resistant colonies and subjected to PCR and Southern blot analysis as previously described (So *et al.*, 2004). Western blot analysis was performed as described (So *et al.*, 2004).

Targeting constructs

TP53 targeting constructs (p53Neo and p53Hyg) were kindly provided by Drs. Vogelstein and Kinzler (The Johns Hopkins Medical Institutions & Howard Hughes Medical Institute). These constructs are designed to replace the first codon of the gene by the first codon of the neomycin- or hygromycin-resistance gene (Bunz *et al.*, 1998) (Fig. 1A). Southern analysis for *TP53* disruption was performed as described previously (Bunz *et al.*, 1998). Targeting constructs and knockout strategies for *XRCC3* have been described earlier (Yoshihara *et al.*, 2004). A promoterless *TDP1* targeting construct carrying a diphtheria toxin A gene will be described elsewhere (Iiizumi *et al.*, in preparation).

Microarray analysis

Total RNA was isolated from logarithmically growing Nalm-6 cells. Two micrograms of total RNA were mixed with 10 pg of λpolyA+RNA-A (Takara Bio Inc., Shiga, Japan) as internal control RNA, labeled with Cy3-UTP, and used for microarray analysis using IntelliGene HS Human Expression CHIP (Takara Bio Inc., Shiga, Japan). Briefly, the labeled probes were mixed with hybridization solution (6 × SSC, 0.2% SDS, 5 × Denhardt's solution, 0.1 mg/ml ssDNA, and 50% formamide). After 14-h hybridization at 70°C, the slides were washed three times in 2 × SSC/0.2% SDS for 10 min at 65°C, and washed briefly in 0.05 × SSC at room temperature. The slides were scanned using the Affymetrix 428 Array Scanner (Affymetrix, Santa Clara, CA), and the signal intensity of hybridization was evaluated photometrically by the ImaGene computer program (BioDiscovery ImaGene Ver.4.2) and normalized to the averaged signals of housekeeping genes (e.g., *ACTB*, *GAPD*, *PRS5*,

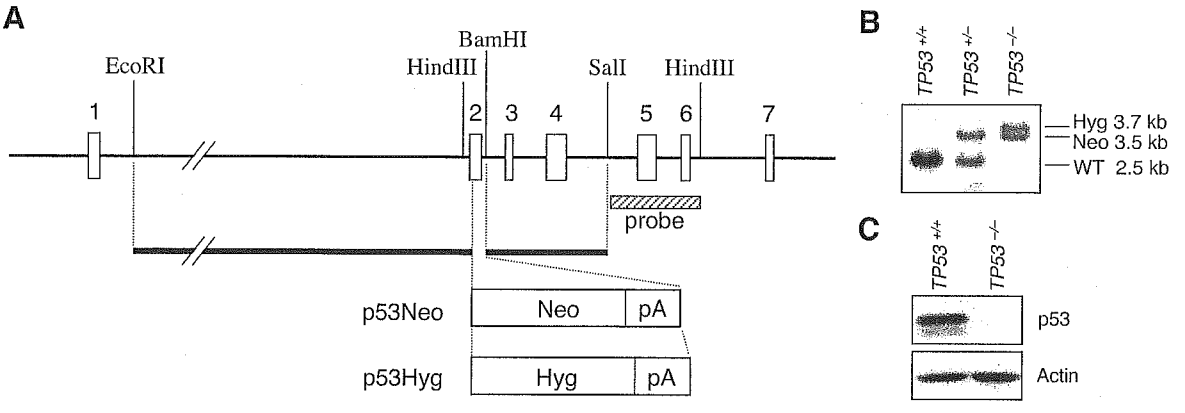

FIG. 1. Targeted disruption of the human *TP53* gene. (**A**) Scheme for *TP53* gene targeting. The human *TP53* gene is composed of 11 exons, located on chromosome 17p13. The *TP53* targeting vectors are designed to replace the first codon of the gene by the first codon of the neomycin- or hygromycin-resistant gene (Bunz *et al.*, 1998). pA, a polyadenylation signalcontaining sequence. (**B**) Southern blot analysis of *Hin*dIII-digested genomic DNA of wild-type (*TP53+/+*), *TP53+/−*, and *TP53−/−* cells. The probe used is shown in (**A**). (**C**) Western blot analysis for p53 protein. Samples used contained 10 μg of total protein extracted from wild-type (*TP53+/+*) and *TP53−/−* cells. Actin served as a loading control.

TABLE 1. SUMMARY OF GENE TARGETING IN NALM-6 AND HCT116 CELL LINES

Cell line	*Targeting construct*	*No. of clones analyzed*	*No. of correctly targeted clones*	*Targeting efficiency*	*Reference*
Nalm-6					
Wild-type	p53Neo	9	1	11%	This study
TP53+/−	p53Hyg	116	9	7.8%[c]	This study
Wild-type	LIG4Neo[a]	124	2	1.6%	Grawunder *et al.*
LIG4+/−	LIG4Puro[a]	20	1	5%[c]	Grawunder *et al.*
Wild-type	XRCC3Neo	6	1	17%	This study
LIG4−/−	XRCC3Hyg	6	1	17%	This study
BLM−/−	p53Neo	1	1	100%	This study
BLM−/− *TP53*+/−	p53Hyg	42	4	9.5%[c]	This study
Wild-type	TDP1Hyg[b]	7	2	29%	This study
HCT116					
Wild-type	p53Neo	600	1	0.16%	Bunz *et al.*
TP53+/−	p53Hyg	940	1	0.11%[c]	Bunz *et al.*
Wild-type	p53Neo	1141	1	0.09%	Traverso *et al.*
BLM−/−	p53Neo	527	8	1.5%	Traverso *et al.*
XRCC3+/−	XRCC3Hyg	193	5	2.6%[c]	Yoshihara *et al.*

[a]Nonpromoterless constructs.
[b]A promoterless construct carrying a DT-A gene.
[c]These values are presumably underestimated because only targeting events on the second allele have been counted.

and *ATP5F1*). The expression profiles (normalized Cy3 intensity values, which roughly reflects the expression level of the gene) of DNA repair genes are summarized in Table 2; those for other genes (~16,500 human genes) are available upon request.

RESULTS AND DISCUSSION

A promoterless targeting construct, p53Neo (Bunz *et al.*, 1998), was transfected into wild-type Nalm-6 cells, and nine G418-resistant (G418^r) colonies were subjected to Southern blot analysis. One of these clones exhibited a band pattern indicative of the precise targeting event (Fig. 1B), indicating that this cell line is heterozygous for the *TP53* gene. Similarly, another promoterless targeting construct, p53Hyg, was transfected into the heterozygous *TP53*+/− cells. A total of 116 hygromycin-resistant (Hyg^r) colonies were screened for homozygous disruption of the *TP53* gene, which allowed us to isolate nine independent *TP53*−/− clones. The disruption of both *TP53* alleles was verified by Southern blot analysis (Fig. 1B) and Western blot analysis using anti-p53 antibody (Fig. 1C). Thus, the targeting efficiencies for *TP53* disruption were 11% for heterozygous knockout and 7.8% for homozygous knockout, as summarized in Table 1. (Note that the latter value is underestimated because the second targeting events on the p53Neo-targeted allele were not taken into consideration.) Remarkably, these values are nearly two orders of magnitude higher than those obtained in the HCT116 colon cancer cell line (Table 1). It should be mentioned that the targeting efficiencies with these promoterless constructs are higher than those reported with non-promoterless constructs in Nalm-6 cells (Grawunder *et al.*, 1998; So *et al.*, 2004) (Table 1). Similar experiments were performed using Nalm-6 derivatives nullizygous for the *LIG4* gene or the *BLM* gene (Table 1). Higher gene-targeting efficiencies were observed with the latter mutant, even though this may not be surprising, given accumulating evidence of enhanced gene targeting in cells lacking BLM protein (Luo *et al.*, 2000; Wang *et al.*, 2000; Traverso *et al.*, 2003).

To further confirm the effectiveness of promoterless constructs, we wished to disrupt another locus in Nalm-6 cells. For this purpose, *XRCC3* was chosen because the gene has already been disrupted in HCT116 cells by using promoterless constructs (Yoshihara *et al.*, 2004). *XRCC3* promoterless constructs were transfected into Nalm-6 cells, and drug-resistant (G418^r or Hyg^r) colonies were screened for heterozygous disruption of the *XRCC3* locus. As expected, the targeting efficiencies were significantly high when compared to HCT116 cells (Table 1). Finally, it would be worth mentioning that a targeting construct for the *TDP1* gene, which was designed to be promoterless as well as to possess a diphtheria toxin A gene, gave a high targeting efficiency of 29% (Table 1), suggesting that a combination of two strategies known to select against random integrants may be useful in further enhancing gene targeting.

Nalm-6 was established from the peripheral blood of a 19-year-old man with acute lymphoblastic leukemia (Hurwitz *et al.*, 1979). Nalm-6 has a stable near-diploid karyotype, though carrying a reciprocal translocation between chromosome 5 and chromosome 12, t(5;12)(q33.2;p13.2) (Wlodarska *et al.*, 1997). The high proficiency of Nalm-6 cells in gene-targeting experiments prompted us to perform genome-wide expression analysis using cDNA microarray. We anticipated that such analysis might help understand the basis of high gene-targeting efficiencies in this cell line. Thus, we focused on expression of DNA repair genes (Table 2), some of which, when inactivated, might potentially affect the efficiency of gene targeting. In fact, we did find that *MSH2* mRNA is barely detectable in Nalm-6

TABLE 2. EXPRESSION PROFILES OF DNA REPAIR GENES IN NALM-6

Gene name	*Accession #*	*GeneID*	*Expression level*
Nonhomologous end-joining			
LIG4	NM_002312	3981	943
XRCC4	NM_003401	7518	254
KU70 (XRCC6)	NM_001469	2547	10121
KU80 (XRCC5)	NM_021141	7520	11272
DCLRE1C (Artemis)	NM_022487	64421	634
Homologous recombination			
RAD51	NM_002875	5888	1437
RAD51C	NM_058216	5889	601
XRCC3	NM_005432	7517	344
RAD54L	NM_003579	8438	1090
RAD54B	NM_012415	25788	269
RAD50	NM_005732	10111	167
MRE11A	NM_005591	4361	91
NBS1	NM_002485	4683	1148
ATM	NM_000051	472	297
BRCA1	NM_007295	672	893
BRCA2	NM_000059	675	1204
SHFM1 (DSS1)	NM_006304	7979	4066
MUS81	NM_025128	80198	1662
EME1 (MMS4L)	NM_152463	146956	642
RUVBL2	NM_006666	10856	11872
Base excision repair/singe-strand break repair			
UNG	NM_080911	7374	823
UNG2	NM_021147	10309	82
SMUG1	NM_014311	23583	422
MBD4	NM_003925	8930	1767
TDG	NM_003211	6996	1032
ALKBH	NM_006020	8846	528
OGG1	NM_016821	4968	207
MUTYH (MYH)	NM_012222	4595	721
NTHL1 (NTH1)	NM_002528	4913	1096
APEX1	NM_001641	328	3325
APEX2	NM_014481	27301	272
LIG3	NM_013975	3980	326
XRCC1	NM_006297	7515	1057
PARP1 (ADPRT)	NM_001618	142	13371
PARP2 (ADPRTL2)	NM_005484	10038	1431
NEIL1	NM_024608	79661	271
NEIL2	NM_145043	252969	641
NEIL3	NM_018248	55247	519
PNKP	NM_007254	11284	807
TDP1	NM_018319	55775	731
APTX (aprataxin)	NM_175073	54840	1262
Direct reversal of base damage			
MGMT	NM_002412	4255	1328
DEPC-1 (ABH3)	NM_139178	221120	1588
Mismatch repair			
MSH2	NM_000251	4436	16
MSH3	NM_002439	4437	414
MSH6	NM_000179	2956	1480
MSH4	NM_002440	4438	135
MSH5	NM_002441	4439	556
MLH1	NM_000249	4292	2121
PMS2	NM_000535	5395	195
MLH3	NM_014381	27030	181
Nucleotide excision repair			
XPC	NM_004628	7508	549
RAD23B (HR23B)	NM_002874	5887	251
CETN2	NM_004344	1069	817
RAD23A (HR23A)	NM_005053	5886	6297
Modulation of nucleotide pools			
NUDT1 (MTH1)	NM_002452	4521	2894
DUT	NM_001025248	1854	4372
RRM2B (p53R2)	NM_015713	50484	131
DNA polymerase (catalytic subunits)			
POLB	NM_002690	5423	345
POLG	NM_002693	5428	1746
POLD1	NM_002691	5424	787
POLE	NM_006231	5426	2615
REV3L (POLZ)	NM_002912	5980	1924
MAD2L2 (REV7)	NM_006341	10459	2929
REV1L (REV1)	NM_016316	51455	169
POLH	NM_006502	5429	304
POLI (RAD30B)	NM_007195	11201	72
POLQ	NM_006596	10721	990
POLK (DINB1)	NM_016218	51426	57
POLM	NM_013284	27434	1727
POLS	NM_006999	11044	1782
Rad6 pathway			
UBE2A (RAD6A)	NM_003336	7319	1041
UBE2B (RAD6B)	NM_003337	7320	403
UBE2V2 (MMS2)	NM_003350	7336	1123
Fanconi anemia			
FANCA	NM_000135	2175	1621
FANCC	NM_000136	2176	239
FANCD2	NM_033084	2177	830
FANCE	NM_021922	2178	458
FANCF	NM_022725	2188	224
FANCG (XRCC9)	NM_004629	2189	1592
FANCL	NM_018062	55120	443
General replication/repair enzymes			
LIG1	NM_000234	3978	1419
RPA1	NM_002945	6117	6434
RPA2	NM_002946	6118	2107
TOP3A	NM_004618	7156	755
TOP3B	NM_003935	8940	297
RECQL (RECQ1)	NM_002907	5965	651
BLM	NM_000057	641	748
BLAP7 (C9orf76)	NM_024945	80010	765
WRN	NM_000553	7486	485
WRNIP1	NM_020135	56897	1696
RECQL4 (RTS)	NM_004260	9401	647
RECQL5	NM_004259	9400	47
HEL308	NM_133636	113510	486
FEN1 (DNase IV)	NM_004111	2237	7501
EXO1 (HEX1)	NM_003686	9156	1398
FLJ35220 (ENDOV)	NM_173627	284131	63
DCLRE1A (SNM1)	NM_014881	9937	164
DCLRE1B (SNM1B)	NM_022836	64858	467
Damage response, chromatin structure			
ATR	NM_001184	545	641
RAD1	NM_002853	5810	128
RAD9A	NM_004584	5883	494
RAD9B	NM_152442	144715	120
HUS1	NM_004507	3364	335
RAD17	NM_002873	5884	676
CHEK1	NM_001274	1111	341
CHEK2 (RAD53)	NM_007194	11200	229
TP53	NM_000546	7157	3057
TP53BP1	NM_005657	7158	488
EEF1E1 (p18)	NM_004280	9521	1075

TABLE 2. EXPRESSION PROFILES OF DNA REPAIR GENES IN NALM-6 (*CONTINUED*)

Gene name	*Accession #*	*GeneID*	*Expression level*	*Gene name*	*Accession #*	*GeneID*	*Expression level*
XPA	NM_000380	7507	720	*BTG2*	NM_006763	7832	224
ERCC3 (XPB)	NM_000122	2071	733	*BACH1 (BRIP1)*	NM_032043	83990	325
ERCC2 (XPD)	NM_000400	2068	389	*MDC1*	NM_014641	9656	1172
GTF2H1	NM_005316	2965	1559	*TOPBP1*	NM_007027	11073	2920
GTF2H2	NM_001515	2966	1405	*H2AFX (H2AX)*	NM_002105	3014	1380
GTF2H3	NM_001516	2967	267	*CHAF1A*	NM_005483	10036	1975
GTF2H4	NM_001517	2968	522	*CHAF1B*	NM_005441	8208	471
CDK7	NM_001799	1022	534	*ASF1A*	NM_014034	25842	729
CCNH	NM_001239	902	1313	*ASF1B*	NM_018154	55723	3175
MNAT1	NM_002431	4331	656	*RAD21 (SCC1)*	NM_006265	5885	4418
ERCC5 (XPG)	NM_000123	2073	995	Meiotic recombination			
ERCC4 (XPF)	NM_005236	2072	272	*DMC1*	NM_007068	11144	39
ERCC8 (CSA)	NM_000082	1161	271	*SPO11*	NM_012444	23626	106
ERCC6 (CSB)	NM_000124	2074	99	Lymphocyte-specific genetic alterations			
XAB2 (HCNP)	NM_020196	56949	275	*TDT (DNTT)*	NM_004088	1791	14980
DDB1	NM_001923	1642	4931	*RAG1*	NM_000448	5896	2587
DDB2	NM_000107	1643	994	*RAG2*	NM_000536	5897	1245
MMS19L	NM_022362	64210	2747	*AID (AICDA)*	NM_020661	57379	138

Microarray gene expression profiling of Nalm-6 was performed using Takara IntelliGene HS Human Expression CHIP. Human DNA repair genes were classified as described (Adachi and Lieber, 2002; Wood *et al.*, 2005), with slight modifications. "Expression level" represents the Cy3 intensity value normalized using control genes and thus roughly reflects the cellular level of the mRNA. Note that the value for *MSH2* is even lower than the values for *DMC1* and *SPO11* (meiosis-specific genes). See "Materials and Methods" for details.

cells. The absence of MSH2 expression in these cells was verified by Western blot analysis using anti-MSH2 antibody (Fig. 2). The MSH2 protein plays a critical role in mismatch repair, an evolutionarily conserved DNA repair system for mismatched bases (Buermeyer *et al.*, 1999; Stojic *et al.*, 2004). As the loss of mismatch repair activity can lead to enhanced gene targeting in yeast and mammals (de Wind *et al.*, 1995; Leung *et al.*, 1997; Elliott and Jasin, 2001; Dekker *et al.*, 2003), the lack of MSH2 expression could account for higher gene-targeting efficiencies. However, HCT116 cells are known to be defective for MLH1, another critical component of mismatch repair (Koi *et al.*, 1994) (see Fig. 2). Thus, the high gene-targeting efficiency in Nalm-6 cannot be explained by the lack of MSH2 expression itself. Further studies will be necessary to clarify this issue.

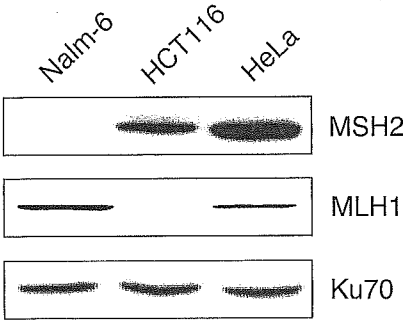

FIG. 2. Lack of MSH2 expression in Nalm-6 cells. Total protein extracted from Nalm-6, HCT116, and HeLa cells was subjected to Western blot analysis for MSH2, MLH1, and Ku70. Note that Nalm-6 cells do not express MSH2, while HCT116 cells are defective for MLH1 expression (Koi *et al.*, 1994). Ku70 served as a loading control.

In conclusion, we have demonstrated that Nalm-6 is highly proficient for gene targeting with the use of promoterless constructs. Although the reason for this has yet to be elucidated, the usefulness of this cell line for gene disruption is quite intriguing and must be emphasized. Recent studies with the avian DT40 B-lymphocyte line have provided a significant contribution to our understanding of cellular functions of a wide variety of vertebrate genes, implying that B-lymphocytes are reliable sources for analyzing gene functions via gene targeting. Additionally, and more importantly, Nalm-6 is a human cell line that is, unlike DT40, normal for p53 status (Filippini *et al.*, 1998). Hence, the results presented here underscore the usefulness of Nalm-6 in the postgenome era for reverse genetic studies of human genes.

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Gene Trap Mutagenesis-based Forward Genetic Approach Reveals That the Tumor Suppressor OVCA1 Is a Component of the Biosynthetic Pathway of Diphthamide on Elongation Factor 2*

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OVCA1 is a tumor suppressor identified by positional cloning from chromosome 17p13.3, a hot spot for chromosomal aberration in breast and ovarian cancers. It has been shown that expression of OVCA1 is reduced in some tumors and that it regulates cell proliferation, embryonic development, and tumorigenesis. However, the biochemical function of OVCA1 has remained unknown. Recently, we isolated a novel mutant resistant to diphtheria toxin and *Pseudomonas* exotoxin A from the gene trap insertional mutants library of Chinese hamster ovary cells. In this mutant, the *Ovca1* gene was disrupted by gene trap mutagenesis, and this disruption well correlated with the toxin-resistant phenotype. We demonstrated direct evidence that the tumor suppressor OVCA1 is a component of the biosynthetic pathway of diphthamide on elongation factor 2, the target of bacterial ADP-ribosylating toxins. A functional genetic approach utilizing the random gene trap mutants library of mammalian cells should become a useful strategy to identify the genes responsible for specific phenotypes.

OVCA1 is a tumor suppressor isolated from chromosome 17p13.3, a hot spot for chromosomal aberration in breast and ovarian cancers (1, 2). It has been shown that expression of OVCA1 is reduced in tumors and that exogenous expression of OVCA1 inhibited growth of ovarian cancer cells (3). Furthermore, a study using *Ovca1* gene knock-out mice clearly showed that OVCA1 regulates cell proliferation, embryonic development, and tumorigenesis (4). Even though the biological or cell biological functions have been elucidated, the biochemical function of OVCA1 has not been ascertained.

Diphthamide is a unique post-translationally modified histidine residue found only on translational elongation factor 2 (EF-2),[1] which catalyzes the translocation of peptidyl tRNA from the ribosome A site to the P site during peptide chain elongation. Diphthamide has been found in all eukaryote and archaebacteria, however not in eubacteria. The diphtheria toxin (DT) and *Pseudomonas* exotoxin A (ETA) inactivate EF-2 by ADP-ribosylating the diphthamide (5, 6). The biosynthesis of diphthamide is one of the most complex post-translational modifications, and by genetic complementation analyses it has been shown that five different genes in yeast (7) and at least three genes in CHO cells (8) are involved in diphthamide synthesis. To date, three genes responsible for diphthamide formation have been elucidated in yeast and human (9–11). The biochemical function of diphthamide as a target of bacterial ADP-ribosylating toxins has been well characterized; however, its physiological role in cells has remained to be clarified.

Bacterial toxins are useful and valuable tools for investigating cell functions; important knowledge concerning cell functions has been obtained by analyses of mutants of established cell lines. Indeed, much of knowledge concerning the mechanisms of toxicity of DT has been elucidated by the study of toxin-resistant mutants (7, 8, 12–18). Although many of the DT-resistant mutants have been isolated, to clarify the genes involved in DT sensitive and/or resistant phenotype was time consuming and sometimes difficult work, especially in mammalian cells.

To increase the efficiency of insertional mutagenesis in mammalian cells, retrovirus gene trap vectors have been developed (19, 20). Gene traps are based on the integration of a reporter gene lacking a promoter into the genome and its expression from a tagged endogenous promoter. When a gene trap vector integrates into expressed genes, insertional mutants can be easily selected by selectable phenotype conferred by the gene trap vector. It is possible to increase the proportion of cells with virus-induced mutations to two to three orders of magnitude higher than in cells containing unselected proviruses by retroviral gene trap selection (21). So far, mutants having a variety of phenotypes have been isolated from CHO cells. This is because CHO cells are hypodiploid and functionally hemizygous at a number of loci (22, 23). For that reason it was expected that a single gene trap insertional mutagenetic event might result in loss of gene functions in CHO cells.

To identify the obligate genes involved in DT sensitivity and/or resistance, including diphthamide biosynthesis, we screened mutants resistant to DT from a random gene trap insertional mutants library of CHO cells. Recently, we have been able to isolate a novel mutant resistant to DT and ETA in which a mutant *Ovca1* gene was disrupted by gene trap mu-

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB194396.

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[1] The abbreviations used are: EF-2, elongation factor 2; DT, diphtheria toxin; ETA, *Pseudomonas* exotoxin A; CHO, Chinese hamster ovary; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RACE, rapid amplification of cDNA ends; RT, reverse transcription.

tagenesis. Here we show genetic and biochemical evidence that the tumor suppressor OVCA1 is a component of the biosynthetic pathway of diphthamide on EF-2, the target of bacterial ADP-ribosylating toxins.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—CHO-K1 cells were obtained from the American Type Culture Collection. CHO-K1 cells and mutants were maintained in Dulbecco's modified Eagle's medium supplemented with 8% fetal calf serum, 2 mM glutamine, and antibiotics (penicillin and streptomycin).

Retroviral Gene Trap Insertional Mutagenesis—The retroviral gene trap vector, ROSAβgeo (20), was used to construct the random gene trap insertional mutants library of CHO cells. To infect the ROSAβgeo to CHO cells, pseudo-retrovirus of ROSAβgeo was produced using a pantropic retroviral expression system (Clontech) according to the manufacturer's instruction. The pROSAβgeo (20) and pVSV-G plasmid constructs were co-transfected to GP293 cells with Lipofectamine (Invitrogen). After 48-h incubation, virus-containing supernatant was harvested, passed through a 0.22-μm filter, and stored at −80 °C until use. For gene trap mutagenesis, CHO-K1 cells were seeded in multiple dishes at 1×10^6 cells per 100-mm dish. After overnight incubation, the medium was replaced with a virus-containing medium. After an additional 48 h, the cells from each dish were re-seeded at 1×10^6 cells per 100-mm dish, and gene trapped mutagenized cells were selected by G418 (200 μg/ml) for 10–14 days. Typically we could get $1–2 \times 10^3$ G418-resistant colonies per dish. Cells from $\sim 1 \times 10^6$ independent colonies were combined and stored as the random gene trap insertional mutants library of CHO cells.

Toxins—DT and fragment A of DT were purified by DEAE-cellulose column chromatography (14). ETA was purchased from List Biological Laboratories.

Selection and Cloning of Diphtheria Toxin-resistant Cells—Toxin-resistant CHO mutants were isolated from the gene trap insertional mutants library. Inocula of 10^6 gene trap mutagenized CHO cells were incubated for 6–10 h before the addition of 1 μg/ml of DT. After about 2 weeks of selection, colonies were isolated with cloning rings, and each isolated clone was cultured in DT-free medium.

Cytotoxicity Assay with MTT—Toxin-induced cytotoxicity was evaluated by conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay. Cells were seeded in 96-microwell plates at a density of 4×10^3/well in Dulbecco's modified Eagle's medium with 8% fetal calf serum. After 16-h incubation, the medium was replaced and cells were exposed to serial dilutions of DT or ETA (0–1000 ng/ml) for 48 h. Then 10 μl of 0.4% MTT reagent and 0.1 M sodium succinate were added to each well. After 90-min incubation, 150 μl of Me_2SO were added to dissolve the purple formazan precipitate. Formazan dye was measured spectrophotometrically (570–650 nm) using the MAXline Microplate Reader (Molecular Devices, Sunnyvale, CA) (11, 24).

Southern Blot Analysis—Genomic DNA was isolated from CHO cells using PUREGENE DNA isolation kits (Gentra), digested with EcoRI, separated by electrophoresis on 0.8% agarose gel, and transferred to a nylon membrane. A 0.8-kbp PCR fragment of the Neo^r gene, amplified with primers 5′-AACCATGGGATCGGCCATTGAACA-3′ and 5′-AGGATCCGCGAAGAACTCGTCAAGAAGGC-3′ from pROSAβgeo, was radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmol) by random priming. The membrane was hybridized with this probe, washed, and then autoradiographed.

5′-Rapid Amplification of cDNA Ends (RACE), Cloning, and Sequence—5′-RACE analyses were conducted using the 5′-Full RACE core set (Takara Biomedicals) with total RNAs from DTR44 cells following the manufacturer's instructions. Total RNAs were prepared from mutant cells with Sepasol RNA I (Nacalai Tesque). Single strand cDNAs were prepared from the total RNAs (~1 μg of total RNA for each sample) with β-galactosidase-specific 5′-phosphorylated RT primer, 5′-ATGCGCTCAGGTCAAATTCA-3′ and avian myeloblastosis virus reverse transcriptase. After the degradation reaction of the hybridized RNAs by RNase H, cDNAs were circularized and/or concatemerized by using the 5′-Full RACE core set following the manufacturer's instructions. For PCR amplification of the trapped sequence, the following β-galactosidase-specific primers sets were used: 5′-GTTGATGAAAGCTGGCTACA-3′/5′-GTGCTGCAAGGCGATTAAGT-3′ (for the first PCR) and 5′-TGATGGCGTTAACTTGGCGT-3′/5′-TTCCCAGTCACGACGTTGTA-3′ (for nested PCR). Amplification products were subcloned to pGEM-T easy vector (Promega) and sequenced by using ABI PRISM™ 377 and 310 sequencing machines (Applied Biosystems). Homology of the trapped sequences was searched by the NCBI BLAST program.

PCR Analyses of Chimera RNA and Genome DNA—Total RNAs were reverse transcribed and amplified by using an RNA PCR kit (Takara Biomedicals). Genome DNAs were prepared from CHO cells using PUREGENE DNA isolation kits (Gentra). For PCR amplification of the cDNA and genome DNA, the following *Ovca1* and β-galactosidase-specific primers sets were used: 5′-CGTTCCTCCAGCGCTGCCTT-3′ (P1)/5′-GTGCTGCAAGGCGATTAAGT-3′ (P3) (for the first PCR) and 5′-TCCAGCGCTGCCTTTTTGGT-3′ (P2)/5′-TTCCCAGTCACGACGTTGTA-3′ (P4) (for nested PCR) (see Fig. 2, *B* and *C*).

cDNA Expression Experiments—The mouse *Ovca1* (*Ovca1* (m)), human *DPH2L2* (*DPH2L2* (h)), and mouse *dph2l2* (*dph2l2* (m)) cDNA expression vectors were constructed as described below. First, the cDNAs used in the expression experiments were RT-PCR amplified from the mouse kidney and human fibroblast mRNAs. The mRNAs were reverse transcribed with oligo dT primer and avian myeloblastosis virus reverse transcriptase. The mouse *Ovca1* cDNA fragments were amplified using 5′-primers for without tag *Ovca1* (5′-ATGCTAGCGT-**GATG**GCGGCGCTGGTTGTGT-3′ (the NheI site is underlined, and the start codon is in boldface)) or for with FLAG-tagged *Ovca1* (5′-ATGC-TAGC**ATG**GCGGCGCTGGTTGTGTC-3′ (the NheI site is underlined and the start codon is in boldface)) and a 3′-primer (5′-ATGGATCCC-CCTGCTGCTCTGGCCCTCTCA-3′ (the BamHI site is underlined)). The amplified cDNA fragments were then digested by NheI and BamHI and cloned into the NheI and BamHI sites of pIREShyg3 (Clontech) or pFLAG-IREShyg3. The pFLAG-IREShyg3 plasmid was constructed as follows. Oligonucleotides, 5′-AGCTTAAGTCCACC**ATG**GATTACAAG-GATGACGAC-3′ (the AflII site is underlined, and the start codon is in boldface) and 5′-TAGCTAGCGATCTTATCGTCGTCATCCTTGTAAT-C-3′ (the NheI site is underlined) were annealed, purified by PAGE, digested by AflII and NheI, and cloned into AflII and NheI sites of pIREShyg3. The mouse *dph2l2* cDNA fragment was amplified using a 5′-primer (5′-ATGCTAGCAAGCTGCGCCC**AATG**GAGTCTA-3′ (the NheI site is underlined and the start codon is in boldface)) and a 3′-primer (5′-CTGATATCCAGACTTATCACTCTGGCTC-3′: the EcoRV site is underlined)). The amplified cDNA fragment was digested by NheI and EcoRV and cloned into the NheI and EcoRV sites of pIRES-Hyg3. The human *DPH2L2* cDNA fragment was amplified using a 5′-primer (5′-CATGGATCCCAAGCTGTGCCTC**ATG**GAGTC-3′ (the BamHI site is underlined and the start codon is in boldface) and a 3′-primer (5′-GATCTCGAGCACATGGTATCAGCCGCTTCC-3′ (the XhoI site is underlined)). The amplified cDNA fragment was digested by BamHI and XhoI and cloned into the BamHI and XhoI sites of pcDNA3.1/Hygro (+) (Invitrogen). These expression plasmid constructs were confirmed by restriction enzyme map and sequencing and were transfected into CHO cells using Lipofectamine reagent (Invitrogen). Stably transfected cells were selected by hygromycin B (400 μg/ml) for 10–14 days. Hygromycin-resistant colonies were isolated and analyzed.

Western Blot Analysis—CHO cells grown in 24-well plates were incubated with or without 1 μg/ml DT at 37 °C for 1 h, and the cells were washed and lysed by 100 μl/ml radioimmune precipitation assay buffer. Cell lysates were separated by native PAGE or SDS-PAGE and transferred to a nylon membrane (Hybond-P, Amersham Biosciences). Membranes were blocked in appropriate blocking buffers and incubated with goat anti EF-2 antibody (sc-13004, Santa Cruz Biotechnology) followed by peroxidase-conjugated anti-goat antibody. Reactive bands were detected by enhanced chemiluminescence (Amersham Biosciences).

In Vitro ADP-ribosylating Assay—CHO cell lysates preparation and ADP-ribosylation reaction of EF-2 were performed as described previously (15, 16). Cell lysates were incubated with [*adenylate*-^{14}C]NAD (Amersham Biosciences, 248 mCi/mmol, catalog no. CFA 497) in the absence or presence of DT fragment A. The amount of ADP-ribosylated EF-2 was assessed by counting the radioactivity incorporated to the acid insoluble fraction in a liquid scintillation counter (Aloka, LSC-3500). ADP ribosylation of EF-2 was confirmed by SDS-PAGE followed by autoradiography.

RESULTS

Isolation of the Diphtheria Toxin-resistant Mutants—The random gene trap insertional mutants library of CHO cells was made by infecting the ROSAβgeo (20) followed by growth in G418 as described under "Experimental Procedures." By combining the cells from $\sim 1 \times 10^6$ independent G418-resistant colonies, we constructed a library of mutants. The gene trap mutagenized cells were inoculated at 20×10^5 cells per

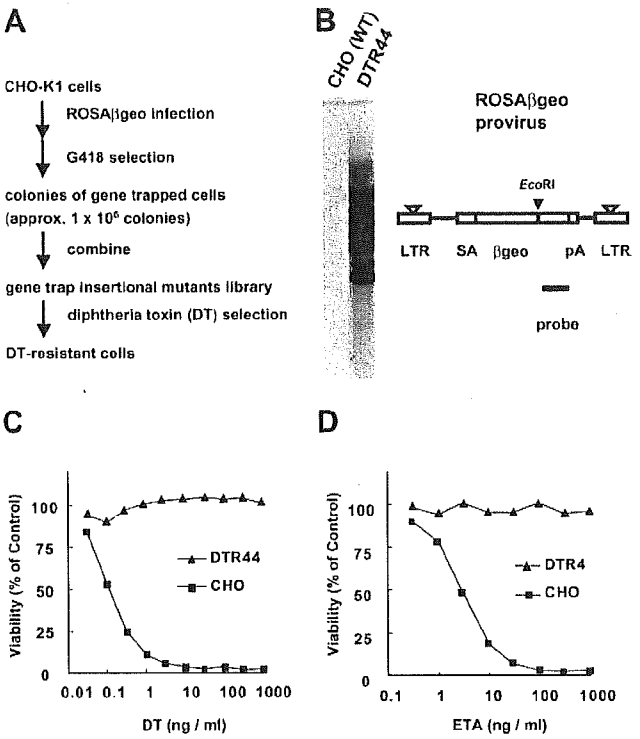

FIG. 1. **DTR44, obtained by retroviral gene trap insertional mutagenesis, is a multiple toxin-resistant mutant.** *A*, schematic representation of the construction of the random gene trap insertional mutants library of CHO cells and the isolation of the DT-resistant mutants. *B*, Southern blot analysis of the retroviral insertions in CHO cells. Genomic DNAs were digested with EcoRI, separated on 0.8% agarose gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled PCR-amplified Neo^r gene fragment. The structure of ROSAβgeo provirus (20) is also shown schematically. *LTR*, long terminal repeat; *SA*, splice acceptor; *βgeo*, β-galactosidase-Neo^r fusion gene; *pA*, polyadenylation signal. Cytotoxicity of DT (*C*) and ETA (*D*) to CHO cells. CHO cells were incubated with various concentrations of DT or ETA. After 48-h exposure to toxins, cell viability was determined by MTT assay as described under "Experimental Procedures."

100-mm culture dish and selected with DT. Colonies, ~10 per dish, were observed 10–14 days later. A total of 24 clones were picked randomly from the 1×10^6 mutant library cells (Fig. 1*A*). One of these mutants, DTR44, was completely resistant to DT and ETA (Fig. 1, *C* and *D*). Southern blot analysis with a Neo^r gene fragment as a probe showed multiple insertions (>8 copies) in the DTR44 genome (Fig. 1*B*).

Identification of the Disrupted Gene in DTR44 Cells—To identify the gene responsible for multiple toxin resistance in DTR44, we amplified the trapped sequences with a gene trap vector, ROSAβgeo, using 5′-RACE. A major PCR product was amplified from the DTR44 cDNA (data not shown) and 128-bp trapped sequences (DDBJ accession number AB194396) were clarified by sequencing. BLAST search revealed that 76 bp (number 53 to 128) of these 128-bp trapped sequences strongly matched those of the first exon of the mouse (25) and human *OVCA1* genes (Fig. 2*A*). The amino acids sequence of Chinese hamster OVCA1 protein in this region completely matched with that of mouse OVCA1, and 85% of these amino acids are identical to those of human OVCA1 protein (Fig. 2*A*).

The existence of the *Ovca1*-βgeo chimera RNA resulting from gene trap mutagenesis was confirmed by RT-PCR of DTR44 mRNA (Fig. 2*B*). Although multiple insertions of the gene trap vector into DTR44 genome were ascertained by Southern blot (Fig. 1*B*), PCR sequence analysis of the DTR44 genomic DNA with the primers correspond to *Ovca1*, and βgeo sequences clarified that one of the gene trap retrovirus was integrated within the first intron, ~1kb downstream of exon 1, of the *Ovca1* gene (Fig. 2*C*).

Disruption of the Ovca1 Gene Renders a Toxin-resistant Phenotype—To define the role of OVCA1 in DTR44, *Ovca1* cDNA expression plasmids were constructed and transfected into DTR44 cells; stable transformants were then established by selecting with hygromycin B (400 μg/ml). Stable transfectant colonies were picked and evaluated for sensitivity to the toxins. The majority of clones arising from DTR44 cells transfected with *Ovca1* cDNA regained sensitivity to DT and ETA (Fig. 3*A*). In contrast, in cells transfected with the empty vector or DPH2L2, proteins that have higher similarity to DPH2 than OVCA1 (26), expression vector remained toxin-resistant (Fig. 3*A*).

Cytotoxicities of DT or ETA to DTR44 cells and the clones of DTR44 transfected with OVCA1 expression constructs were further analyzed by MTT assay. Clones R1 and R2, DTR44 stable transformants of mouse *Ovca1* cDNA expression vector, regained their parental sensitivity and were fully sensitive to DT and ETA (Fig. 3, *C* and *D*).

The presence of *Ovca1*-βgeo chimera RNA in these transformants (Fig. 3*B*) clearly showed that the recovery of toxin sensitivity was not caused by the deletion of the gene trap vector integrated in the *Ovca1* gene. Taken together, we concluded that OVCA1 was required for the process involved in sensitivity to DT and ETA.

OVCA1 Is Required for Diphthamide Biosynthesis—DTR44 cells showed the characteristics of the DT^{R}II phenotype, that is, multiple toxin resistances and a resistant to high concentrations of toxins. DT^{R}II mutants have shown that DT sensitivity was affected at the level of EF-2 (8, 12, 13, 15–18).

To determine whether DTR44 cells are altered in their susceptibility of EF-2 to ADP-ribosylation, CHO cell extracts were assayed for transfer of radiolabeling from NAD$^+$ to EF-2. Lysates were incubated with [*adenylate*-^{14}C]NAD in the absence or presence of DT fragment A. The amount of ADP-ribosylated EF-2 was assessed by counting the radioactivity incorporated to the acid insoluble fraction with a liquid scintillation counter (Fig. 4*A*). ADP ribosylation of EF-2 was confirmed by SDS-PAGE followed by autoradiography. (Fig. 4*B*).

ADP-ribosylation assay clearly showed that ADP-ribosylated acceptor activity in DTR44 cell lysate was dramatically reduced compared with that of wild type parental CHO cells (Fig. 4, *A* and *B*). Furthermore, the non-ribosylatable EF-2 from DTR44 cells restored the ADP-ribosyl acceptor activity when transfected with OVCA1 expression vector (Fig. 4, *A* and *B*). From these observations, we concluded that DTR44 cells are defective in diphthamide formation on EF-2 and that OVCA1 is required for the biosynthesis of diphthamide.

Identification of Intermediate in Diphthamide Synthesis—ADP-ribosylated, the non-ADP-ribosylated form, and biosynthetic intermediate of diphthamide on EF-2 have been shown to be easily distinguished by native PAGE followed by Western blotting using an anti-EF-2 antibody (11). Using this detection system, we examined the diphthamide biosynthetic intermediate in DTR44 cells. ADP-ribosylated EF-2 has two added negative charges compared with EF-2, and this increased negative charge could be detected as faster migration on native PAGE. The EF-2 of DTR44 migrates between the ADP-ribosylated and non-ADP-ribosylated forms (Fig. 4*C*). This observation further confirmed that OVCA1 is required for the biosynthesis of diphthamide on EF-2, the target site for the ADP-ribosylating bacterial protein toxins.

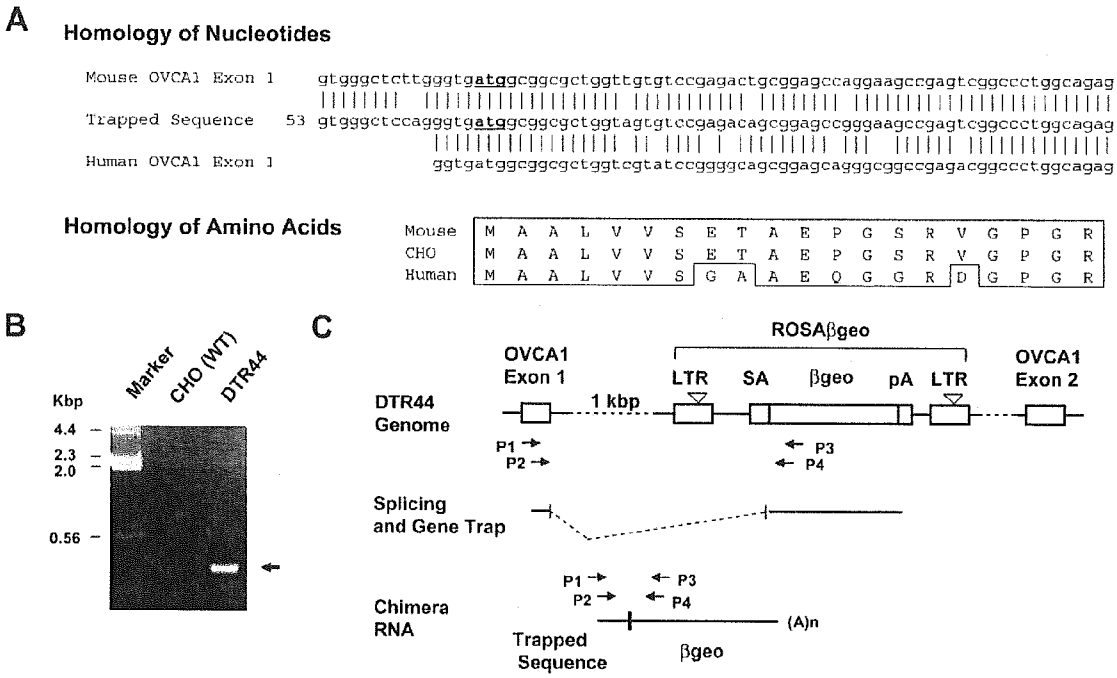

FIG. 2. **The tumor suppressor *Ovca1* gene was disrupted by gene trap insertional mutagenesis in DTR44 mutant.** *A*, the nucleotides sequences (DDBJ accession number AB194396) trapped by ROSAβgeo in DTR44 cells were elucidated by 5′-RACE and sequence analyses of DTR44 cDNA. BLAST homology search revealed that the first exon of *Ovca1* gene was trapped in DTR44 mutant cells. Homologies of the nucleotides and the deduced amino acids of mouse, Chinese hamster (CHO cells), and human OVCA1 in this region are shown. *B*, *Ovca1*-βgeo chimera RNA produced by gene trap event was confirmed in DTR44 mutant cells by RT-PCR as described under "Experimental Procedures." The *arrow* indicates the 211-bp RT-PCR (nested PCR) product amplified using primers P2 and P4 from *Ovca1*-βgeo chimera-RNA in DTR44 cells. *C*, schematic representation of gene trap event in the *Ovca1* gene of DTR44 cells. Gene trap retrovirus was integrated in the first intron of *Ovca1* gene. PCR-sequence analyses of the DTR44 genomic DNA with the primers corresponding to *Ovca1* and βgeo sequences (P1/P3 and P2/P4) clarified that one of the gene trap retrovirus was integrated in the first intron, ~1kb downstream of exon 1, of the *Ovca1* gene (data not shown).

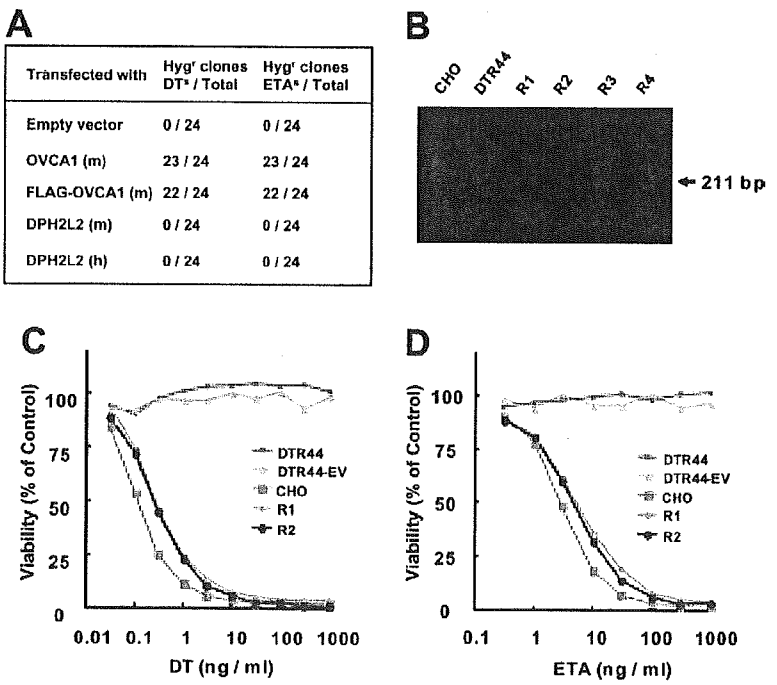

FIG. 3. **Expression of OVCA1 restores the sensitivity of DTR44 mutant cells to bacterial ADP-ribosylating toxins.** *A*, effect of OVCA1 or DPH2L2 expression on sensitivity to toxins. The expression plasmids of the indicated genes, mouse *Ovca1* (*OVCA1* (m)), FLAG-tagged mouse *Ovca1* (FLAG-*OVCA1* (m)), mouse *dph2l2* (*DPH2L2* (m)), and human *DPH2L2* (*DPH2L2* (h)) were transfected to DTR44 mutant cells, and stably transformed cells were established through growth for 10–14 days in hygromycin B (400 μg/ml). The cell colonies were isolated and subcultured to 48-well plates and then treated with DT (1 μg/ml) or ETA (1 μg/ml) for 96 h. The numbers of toxin-sensitive mutants (DT^s, DT-sensitive; ETA^s, ETA-sensitive) *versus* those that tested the sensitivities to toxins were determined by microscopic observation. *B*, RT-PCR analysis of the *Ovca1*-βgeo chimera RNA. *Ovca1*-βgeo chimera RNA was examined in CHO cells as in Fig. 2*B*. R1 to R4 are DTR44 cells transfected with mouse OVCA1 expression constructs and confirmed the recovery of sensitivity to toxins. R1 and R2 clones were used for further analyses. Cytotoxicities of DT (*C*) or ETA (*D*) to CHO cells were determined by MTT assay as in Fig. 1. DTR44-EV is a stably transformed DTR44 cells transfected with an empty vector, pIREShyg3.

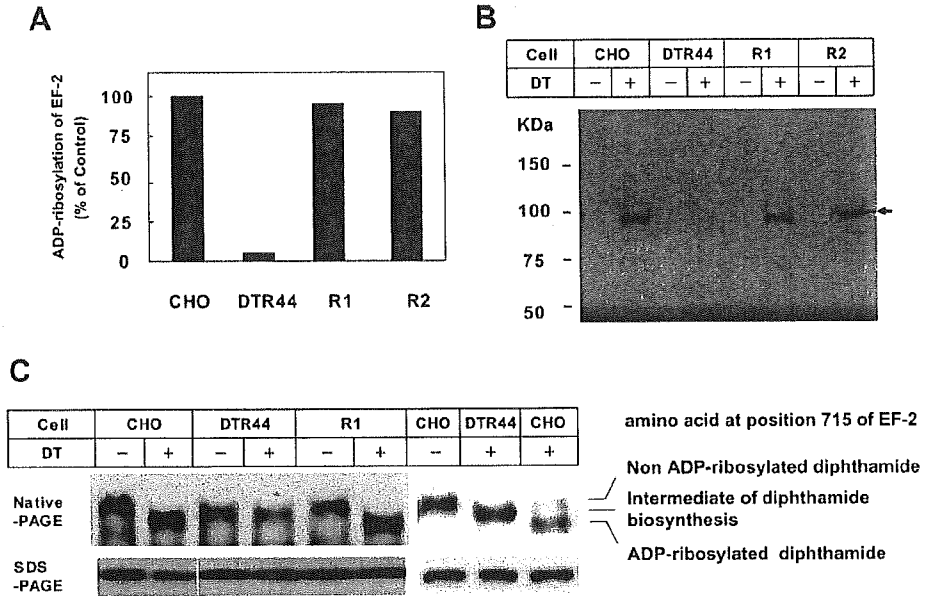

FIG. 4. **DTR44 mutant cells are defective in diphthamide formation of EF-2.** CHO cell lysates were incubated with [*adenylate*-^{14}C]NAD in the absence or presence of DT. *A*, *in vitro* ADP-ribosylating assay. EF-2 of DTR44 cells is resistant to ADP-ribosylation by DT. The amount of ADP ribosylated EF-2 *in vitro* was assessed by counting the radioactivity incorporated to the acid insoluble fraction as described previously (15, 16). R1 and R2 are DTR44 clones transfected with mouse OVCA1 expression vector. *B*, ADP-ribosylation of EF-2 *in vitro* was confirmed by SDS-PAGE followed by autoradiography. One major band (~95 kDa) corresponding to ADP-ribosylated EF-2 was observed. EF-2 of CHO, R1, and R2 cells were the ADP-ribosylatable form. The non-ribosylatable EF-2 in DTR44 cells could be converted to the ADP-ribosylatable form by transfection of cells with OVCA1 expression vector. *C*, Western blots analyses of the EF-2. After 1 h incubation with or without 1 μg/ml of DT at 37 °C CHO cells were lysed by radioimmune precipitation assay buffer, and cell lysates were separated by native PAGE (*top*) or SDS-PAGE (*bottom*) followed by Western blots analyses using an anti-EF-2 antibody as described under "Experimental Procedures."

DISCUSSION

To elucidate the molecular mechanisms underlying DT sensitivity and/or resistance, including diphthamide biosynthesis, we screened mutants resistant to DT from a library of random gene trap insertional mutants of CHO cells. CHO cells are functionally hemizygous at a number of loci (22, 23). It was expected that a single integration event of gene trap retrovirus might result in the loss of gene function in CHO cells, and we would be able to get mutants with specific phenotype by proper selection from this library of mutant cells.

DTR44 mutant cells isolated from the gene trap insertional mutants library showed a phenotype with multiple toxin resistance to DT and ETA. It was revealed that the *Ovca1* gene was disrupted by gene trap mutagenesis and that the expression of OVCA1 fully recovered the sensitivity to toxins and EF-2 diphthamide formation. These genetic and biochemical data clearly show that the tumor suppressor OVCA1 is a component of the biosynthetic pathway of diphthamide on EF-2.

So far the amino acid sequences of OVCA1 did not reveal any functional protein domains suggesting its biochemical function except for low level sequence similarity (~20%) (1, 2) between OVCA1 and DPH2, a yeast protein necessary for diphthamide formation (10). The forward genetic approach using gene trap mutagenesis in this study clearly demonstrated the biochemical function of OVCA1 in the formation of diphthamide in mammalian cells as described above.

The retroviral gene trap mutagenesis approach described in this study is relatively simple and straightforward. Even though multiple copies of gene trap vectors are integrated into DTR44 mutant genome (Fig. 1*B*), we could easily identify the obligate gene for DT-resistant phenotype in DTR44 cells by analyzing the trapped sequences in the chimera RNA produced by gene trap mutagenesis. Retroviruses can be used as insertional mutagens to isolate specific genes in mammalian cells. However, in practice, conventional retroviruses are inefficient mutagens (27). In ROSAβgeo, the retroviral gene trap vector used in this study, βgeo is flanked by an upstream 3′-splice consensus sequence (splice acceptor) and a downstream polyadenylation site to ensure its activation from integrations into introns ("intron trap"), and the gene trap events by SAβgeo are estimated to ~4.5 to 11.6% of integration events (20). So even if multiple integration events occurred, it was estimated that a large portion of retroviral vector integrations were not involved in the specific phenotypes.

After the isolation of the *Ovca1* gene (1, 2), the biological functions of OVCA1 were clarified by cell biological analyses and the study of gene knock-out mice. It was speculated that the loss or haploinsufficiency of OVCA1 might be an important event in ovarian tumorigenesis from the observation that expression of OVCA1 protein in ovarian tumor tissues or cell lines was reduced (3). It was also seen that exogenous expression of OVCA1 in ovarian cancer cells causes suppression of cell growth with an increased number of cells in G_1 phase of the cell cycle, suggesting that OVCA1 may play a role in the control of cell cycle/cell growth (3). Furthermore, study using knock-out mice demonstrated that OVCA1 regulates cell proliferation, embryonic development, and tumorigenesis (4).

The fact that the tumor suppressor OVCA1 is involved in diphthamide biosynthesis on EF-2 suggests the possibility that aberrations in translational regulation may be one of the molecular mechanisms underlying the tumorigenesis caused by the defect of OVCA1. So far it has been elucidated that components of the protein synthesis apparatus seem to be involved in the control of cell proliferation, and aberrations in protein synthesis are commonly encountered in established cancers (28). Furthermore, it has been demonstrated that removal of regulation of the expression of components of the translational machinery, such as elongation factor-1 α, a GTP-binding protein that catalyzes the binding of aminoacyl-transfer RNAs to

the ribosome, predispose cells to become more susceptible to malignant transformation (29).

It has also been demonstrated that the activity of EF-2 kinase was markedly increased in several forms of malignancies and that inhibition of EF-2 kinase inhibited the growth of a variety of cancer cell lines (28, 30–32). Phosphorylation of EF-2 by EF-2 kinase results in a drastic inhibition of protein synthesis, and dephosphorylation of EF-2 by phosphatase restores its activity. The phosphorylation of EF-2 directly affects the elongation stage of translation, and this represents a novel mechanism of translational control (33). It is possible to speculate that the defect of OVCA1 also disturbs the translational regulation through the abnormal diphthamide formation on EF-2 and results in the cause of tumorigenesis.

The fact that OVCA1 is a component of diphthamide biosynthetic pathway may also provide an important clue for a better understanding of the biological function of diphthamide. The biosynthesis of diphthamide represents one of the most complex post-translational modifications of an amino acid known to date and is widely well conserved (5, 6), suggesting that it has real importance for biological function. However, the function and role of diphthamide in cellular physiology still remains obscure.

Thus far, the existence of endogenously ADP-ribosylated EF-2 and cellular ADP-ribosyltransferase activity has been found in a variety of animals and tissues. The enzyme transfers ADP-ribose from NAD to elongation factor 2, inactivating the factor like bacterial toxins (34–36). However, the nature of the cellular ADP-ribosyltransferase and its physiological significance remain unknown. To clarify that the effect on cell proliferation, embryonic development, and tumorigenesis observed in the *Ovca1* knock-out mice (4) truly result from the defect of diphthamide, the generation of mice lacking other genes in the diphthamide biosynthesis may be helpful.

The finding that OVCA1 is a component of the diphthamide synthetic pathway will shed light for the further understanding of the function of OVCA1, molecular mechanisms underlying the tumorigenesis in the defect of OVCA1, and the physiological role of diphthamide. Furthermore, a functional genetic approach utilizing the random gene trap mutants library of CHO cells described above should become a useful strategy to identify the genes responsible for specific phenotypes.

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