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High Level, Regulated Expression of the Chimeric P-Enolpyruvate Carboxykinase (GTP)-Bacterial O^6-Alkylguanine-DNA Alkyltransferase (*ada*) Gene in Transgenic Mice[1]

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ABSTRACT

Transgenic animals expressing genes capable of repairing DNA may be a valuable tool to study the effect of DNA-damaging agents on tissue-specific carcinogenesis. For this reason, we constructed a chimeric gene consisting of the promoter-regulatory region of the phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (PEPCK) gene linked to the *Escherichia coli ada* gene coding for O^6-alkylguanine-DNA alkyltransferase and the polyadenylate region from the bovine growth hormone gene. The PEPCK promoter results in gene expression in liver and kidney and is induced by hormones, and its transcription is regulated by diet. The chimeric PEPCK *ada* gene was injected into the male pronucleus of fertilized eggs to produce transgenic mice. Six of 65 developing mice contained 5–10 copies of the intact *trans* gene per genome. Two founders transmitted the *trans* gene in a heterozygous manner, whereas 3 transmitted as germ line mosaics and 1 did not transmit to F_1 offspring. All F_1 offspring carrying the PEPCK *ada trans* gene expressed *ada* mRNA in liver and kidney and produced a functional alkyltransferase with a protein molecular weight of 39,000 originating from the bacterial gene. Total alkyltransferase activity was increased in the liver of F_1 offspring from all founder mice, but offspring of only one founder had elevated renal alkyltransferase levels. A diet high in protein markedly increased *ada* mRNA and alkyltransferase activity within 1 week in both liver and kidney, whereas a high carbohydrate diet for 1 week markedly reduced expression of PEPCK *ada* and alkyltransferase levels. Nontransgenic animals were unaffected by these dietary manipulations. During induction with a high protein diet, hepatic alkyltransferase in transgenic mice was 16.6 ± 1.5 units/μg DNA (mean ± SE) compared to 5.3 ± 0.6 units/μg DNA in control animals. This level of alkyltransferase is higher than that in any mammalian tissue noted previously except human liver. Transgenic animals expressing high levels of alkyltransferase should help define the role of DNA repair in protection from carcinogenesis induced by *N*-nitroso compounds.

INTRODUCTION

The tissue specificity of chemical carcinogenesis appears to depend on the overall level of DNA damage relative to the capacity for DNA repair. Perhaps the best studied example of this is the carcinogenicity of the *N*-nitroso compounds which damage DNA and induce tumors in specific rat and mouse tissues (1–3). One of the critical proteins responsible for repair of DNA damage induced by *N*-nitroso compounds is O^6-alkylguanine-DNA alkyltransferase (alkyltransferase) which repairs O^6-alkylguanine lesions in double-stranded DNA (4, 5). Thus, compounds which directly damage DNA, such as the nitrosoureas, induce tumors in tissues which have a low capacity for repair of O^6-alkylguanine-DNA adducts (*e.g.*, brain, mammary tissue, bone marrow and thymus (6–9). Other *N*-nitroso compounds, such as the nitrosamines, require metabolic activation to produce the proximate carcinogen (10). With these agents, tissues which enzymatically produce the proximate carcinogen, such as liver, kidney, and lung, are the targets for malignant transformation (11, 12). In many instances, the target tissues for nitrosamine carcinogenesis have higher alkyltransferase activity than brain, mammary tissue, or bone marrow but are able to activate the nitrosamine and thus develop high levels of DNA damage (11–13). In these tissues, carcinogenic doses are those that produce sufficient O^6-alkylguanine lesions to overwhelm the alkyltransferase (1, 3, 11–13). While it appears that the alkyltransferase has an important role in protection from *N*-nitroso compound induced carcinogenesis, it is also clear that the relationship between DNA damage and tissue specific DNA repair in chemical carcinogenesis is complex. For this reason, it is important to analyze the carcinogenic process within defined systems where manipulation of a single component of the process can be studied.

Previous studies have documented that alterations in alkyltransferase activity can change cellular susceptibility to *N*-nitroso compounds *in vitro*. Decreasing the level of alkyltransferase in tissue culture cells using the specific alkyltransferase inhibitor, O^6-methylguanine, increases the cytotoxicity, mutagenicity, and chromosomal aberrations caused by both methylating and chloroethylating nitrosoureas (14–16). To significantly increase alkyltransferase activity in mammalian cells, we and others have used gene transfer of the *Escherichia coli* alkyltransferase gene, *ada* (17–19). The bacterial alkyltransferase repairs O^6-methylguanine adducts by a similar mechanism to that of the mammalian protein (20). Following *ada* gene transfer into mammalian cells, expression of the bacterial alkyltransferase results in resistance to the cytotoxicity and chromosomal aberrations of nitrosoureas (17–19). Cumulatively, the *in vivo* and *in vitro* data suggest that the alkyltransferase protects cells from the DNA-damaging effects of *N*-nitroso compounds. However it remains unknown whether increased expression of alkyltransferase could alter the carcinogenicity of *N*-nitroso compounds and decrease tumor induction in a tissue-specific manner.

Transgenic animals expressing DNA repair genes may be a valuable tool to define the effect of DNA-damaging agents on tissue-specific carcinogenesis. For this reason, we produced transgenic animals expressing the *ada* gene. To provide tissue specificity of *ada* gene expression, we utilized the inducible and tissue-specific mammalian promoter, PEPCK[3] (EC 4.1.1.32) (21, 22) to potentially target gene expression to the liver and

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[3] The abbreviations used are: PEPCK, phosphoenolpyruvate carboxykinase (GTP); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; poly(A), polyadenylate.

kidney. The PEPCK promoter is a well-characterized and unique promoter system which has defined glucocorticoid and cAMP response elements (23). Transcription from the PEPCK promoter is increased in the presence of glucocorticoids and cAMP and decreased by insulin in tissue culture cells (24, 25). In transgenic animals, the PEPCK promoter acts in the manner predicted by expression of endogenous PEPCK: when linked to the bovine growth hormone gene, the chimeric *trans* gene is efficiently expressed in a tissue-specific manner in liver and kidney and is regulated by diet and hormones (22). By utilizing the chimeric PEPCK *ada* gene, our aim was to target *ada* expression to liver and kidney and determine whether alkyltransferase activity in these tissues can be induced significantly above basal levels. Characterization of the expression and inducibility of the chimeric gene will enable us to then test the hypothesis that increased alkyltransferase activity will decrease carcinogenesis of *N*-nitroso compounds *in vivo*.

MATERIALS AND METHODS

Chemical Reagents. Restriction endonucleases and other DNA-modifying enzymes were purchased from Boehringer Mannheim. The oligonucleotide-labeling kit was obtained from Pharmacia. [α-^{32}P]dCTP (3000 Ci/mmol) and Gene Screen Plus were obtained from New England Nuclear. Geneticin (G-418) was purchased from GIBCO. Guanidinium thiocyanate was obtained from Fluka and cesium chloride from Boehringer Mannheim. All of the other reagents used in the study were purchased from Sigma Chemical Co. or Pharmacia and were of the highest purity available.

Animal Maintenance and Breeding. Animals were fed a regular diet of Purina rodent chow and acidified water *ad libitum* and given standard fluorescent light illumination for a 12-h cycle each day. Certain animals were switched to either the high protein or high carbohydrate diet formula for either 1 or 4 weeks. Animals were killed by cervical dislocation while under ether anesthesia. The high protein diet contained 64% casein, 22% α-cell nutrient fiber, 11% vegetable oil, 2% brewers' yeast, and a 1% mineral mix with vitamins. The high carbohydrate diet contained 81.5% sucrose, 12.2% casein, 0.3% DL-methionine, 4% cotton seed oil, 2% brewers' yeast, and a 1% mineral mix plus vitamins (Nutritional Biochemical Corp.).

Plasmids. pSV2*adaalkB* was kindly supplied by L. Samson, Harvard School of Public Health (19), and pBSM13 into which the 3′ end of the bovine growth hormone gene had been inserted was the gift of F. Rottman, Case Western Reserve University School of Medicine. pPCKBH1.2 has been described (24). The 1.3-kilobase rat GAPDH complementary DNA fragment was isolated from pRGAPDH-1 (26).

Plasmid Construction and Preparation of Recombinant DNA for Injection. The *E. coli* alkyltransferase gene, *ada*, was isolated as the 1320-base pair *Hind*III-*Sma*I fragment from pSV2*adaalkB*. The pSV2*adaalkB* plasmid contains the *ada-alkB* operon which was originally isolated by Lemotte and Walker (27) from *E. coli* K-12. The 621-base pair *Bam*HI-*Bgl*II fragment isolated from pPCK BH1.2 contains the region from −548 to +73 of the rat PEPCK promoter-regulatory region. The PEPCK promoter and *ada* gene fragments were ligated together in the Cla12N plasmid (28) and isolated following *Hind*III-*Sma*I digestion (Fig. 1). The 710-base pair *Sma*I-*Eco*RI fragment from the 3′ end of the bovine growth hormone gene which includes a portion of the fifth exon and the poly(A) region (29) was ligated into pBS M13,[4] and the PEPCK *ada* chimeric gene was then inserted 5′ to the bovine growth hormone fragment at the *Hind*III-*Sma*I sites. The 2340-base pair *Pst*I-*Sph*I fragment which contains the PEPCK *ada* gene and 364 base pairs from the 710-base pair region of the bovine growth hormone gene [which includes the poly(A) sequences] was isolated by agarose gel electrophoresis, purified by phenol-chloroform extraction, passaged through an Elutip filter (Schleicher and Schuell), and suspended in sterile water for injection at 15 ng/μl.

Microinjection of Recombinant DNA into Single-Cell Embryos. The procedure for microinjection of recombinant DNA has been described in detail (30). Briefly, the fertilized embryo was flushed from C57B/6XSJL F_1 mice 8 h after ovulation, and the pronucleus was injected with 2 pl of DNA solution containing PEPCK *ada* after which the embryo was reimplanted in pseudopregnant mice (30). Offspring were weaned at 4 weeks and tail samples taken for analysis of DNA.

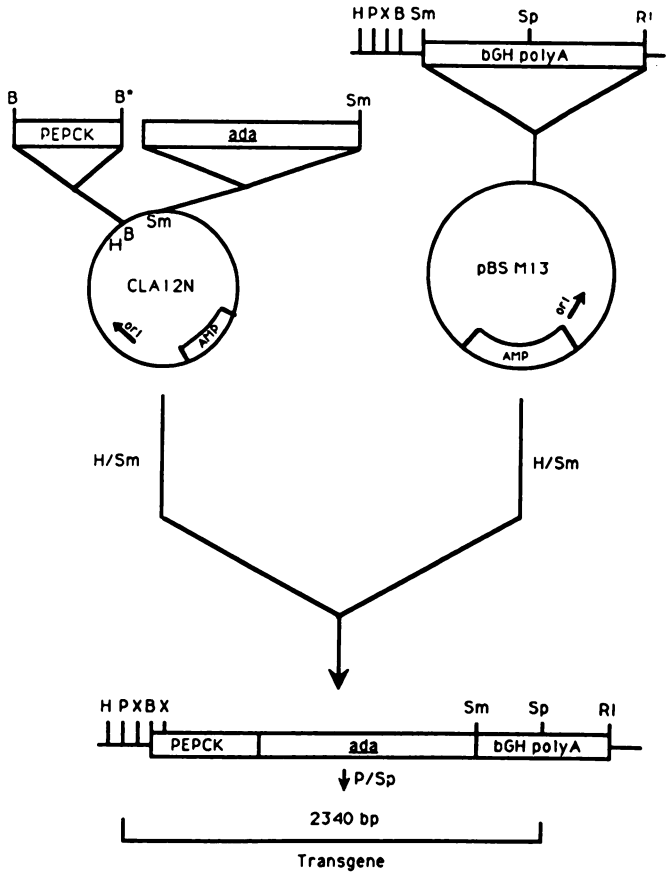

Fig. 1. Construction of the chimeric PEPCK *ada* gene. The 621-base pair *Bam*HI-*Bgl*II PEPCK promoter-regulatory region from pPCK BH1.2 was ligated to the 1320-base pair *Hind*III-*Sma*I *ada* gene from pSV2*adaalkB* in the Cla12N plasmid. The 710-base pair *Sma*I-*Eco*RI fragment containing the 3′ end of the bovine growth hormone gene including the poly(A) region (29) was inserted into the pBS M13 plasmid. The PEPCK *ada* gene was isolated following *Hind*III-*Sma*I digestion and ligated 5′ to the bovine growth hormone fragment in pBS M13. The 2340-base pair *Pst*I-*Sph*I fragment containing PEPCK *ada* and 364 base pairs of the bovine growth hormone gene poly(A) signal was isolated and used for microinjection to produce the transgenic animals. *B*, *Bam*HI; *B**, *Bgl*II; *H*, *Hind*III; *P*, *Pst*I; *RI*, *Eco*RI; *Sp*, *Sph*I; *Sm*, *Sma*I; *X*, *Xba*I; *bGH*, bovine growth hormone; *bp*, base pairs.

DNA Analysis. Mouse tissues were removed and dissected over liquid nitrogen and immediately frozen in liquid nitrogen and stored at −80°C. The general methods used to identify mice carrying the *trans* genes have been previously reported (22, 30). Briefly, DNA was extracted from the tall as described (22, 31), resuspended in 10 mM Tris, pH 7.5, and 1 mM EDTA and blotted onto Gene Screen Plus filter paper under vacuum at concentrations of 2.5, 5, and 10 μg using a Schleicher and Schuell slot blot manifold. The number of gene copies per animal was estimated by standardizing each slot blot with known amounts of plasmid DNA containing the *ada* gene. The gene copies per founder are given as a range of values obtained from the founder and F_1 offspring, when available.

For slot blot analysis, the Gene Screen Plus filter was baked for 2 h in a vacuum oven, prehybridized in a solution of 50% formamide, 0.25 M Na_2HPO_4, 0.25 M NaCl, 2 mM EDTA, 0.1% dried milk, 7% SDS, and 100 μg/ml denatured salmon testes DNA for 2 h at 42°C, and probed with the 1.3-kilobase *Hind*III-*Sma*I *ada* fragment isolated from pSV2*adaalkB* (labeled with [α-^{32}P]dCTP using the Pharmacia random primer kit). The filters were incubated for 48 h at 42°C, washed in 0.1× standard saline citrate-0.1% SDS at 50°C for 45 min, and exposed to

[4] F. Rottman, unpublished results.

x-ray film at −70°C. To determine the size of the integrated chimeric *trans* gene, 20 μg genomic DNA was digested with either *Xba*I or *Pst*I and *Sph*I at 5 units enzyme/μg DNA for 16 h at 37°C and separated on a 0.8% agarose gel and analyzed by Southern blotting as previously described, using the conditions described above.

RNA Analysis. Animals were killed by cervical dislocation and dissected tissues were immediately frozen in liquid nitrogen and then homogenized in 4 M guanidinium hydrochloride, and the RNA was isolated following cesium chloride centrifugation (31, 32). Total cellular RNA (20 μg) was separated on a 1% agarose gel containing 0.66 M formaldehyde (33), transferred to Gene Screen Plus, cross-linked to the membrane with UV light for 3 min, and baked for 2 h at 80°C. The membrane was hybridized with the 1.3-kilobase *ada* probe (see above) in buffer consisting of 50% formamide, 1 M NaCl, 0.1% $NaHPO_2$, 0.2% bovine serum albumin, 0.2% Ficoll, 0.1% SDS, and 100 μg/ml salmon testes DNA. The membranes were hybridized at 42°C for 48 h, washed with 0.1× NaCl-sodium citrate buffer-0.1% SDS at 55°C for 30 min, and exposed as described above for Southern analysis. Each membrane was washed with 1% glycerol for 3 min at 80°C to remove *ada* probe and rehybridized with GAPDH probe to identify the variability of mRNA loading in each lane.

Measurement of O^6-Alkylguanine-DNA Alkyltransferase. Tissues were dissected from animals and frozen in liquid nitrogen. To prepare tissue extract, tissues were suspended at 100 mg/ml in cell extract buffer [70 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.1 mM EDTA, 5% glycerol, and 1 mM dithiothreitol, pH 7.8], homogenized, and sonicated (34). The activity of alkyltransferase in each cell extract was measured as removal of the methyl-^{3}H adduct from O^6-[^{3}H] methylguanine in methyl-^{3}H DNA alkylated with *N*-[^{3}H]methylnitrosourea (4, 34, 35). An alkyltransferase unit of activity is defined as the removal of 1 fmol of O^6-methylguanine from the substrate DNA and is reported as units/μg cellular DNA (35).

SDS-PAGE Analysis of Alkyltransferase. Reaction of the alkyltransferase with substrate [methyl-^{3}H]DNA-containing O^6-[^{3}H]methylguanine adducts results in covalent transfer of the methyl-^{3}H group to the alkyltransferase. Following SDS-PAGE, the two forms of the alkyltransferase can easily be distinguished by molecular weight: the molecular weight of bacterial alkyltransferase is 39,000 with two proteolytic active products (M_r = 20,000 and 19,000) and the molecular weight of the mammalian protein is approximately 23,000 (36). Tissue extract containing 1 mg protein was reacted with 15 μg methyl-^{3}H DNA (specific activity 10.4 dpm/fmol O^6-methylguanine) at 37°C for 45 min. The reaction mixture was separated by 11% SDS-PAGE (17) and soaked in Enhance (New England Nuclear) for 1 h at 22°C, and an autoradiogram was exposed.

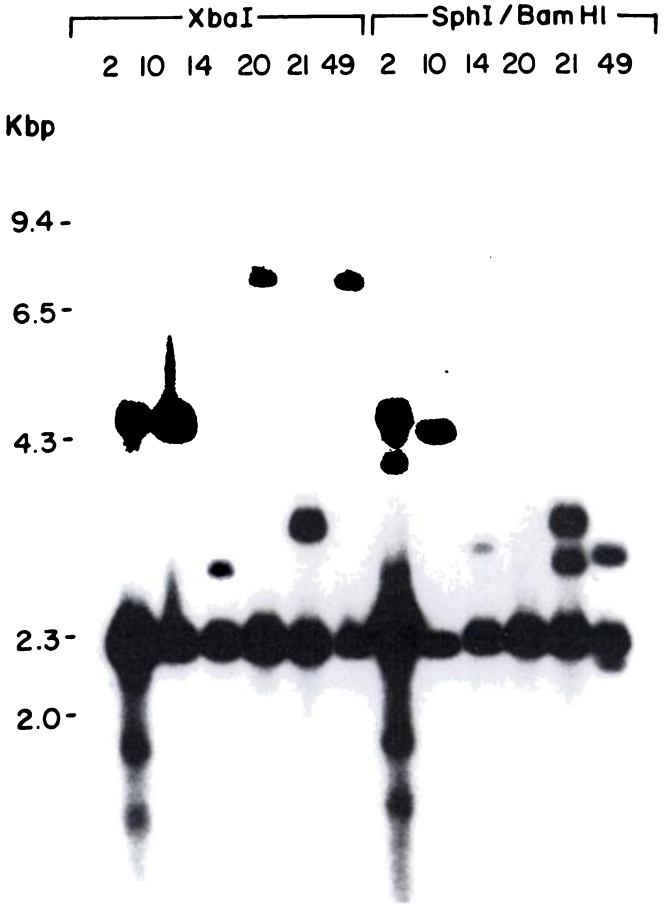

Fig. 2. Identification of the PEPCK *ada trans* gene in founder transgenic mice. Twenty μg genomic DNA prepared from the tail of founder animals were digested with *Sph*I and *Bam*HI to excise the intact *trans* gene or with *Xba*I which cuts once within the *trans* gene to identify tandem repeats and *trans* gene/genomic DNA junctional fragments. The DNA was separated on a 0.8% agarose gel and subjected to Southern analysis using the intact *ada* gene as probe. The expected size of the *trans* gene is 2.3 kilobase pairs (*Kbp*).

RESULTS

Production of Founder Animals. The PEPCK *ada* gene (Fig. 1) was injected into the pronuclei of multiple embryos after which 65 normal mice were born. Southern analysis of genomic DNA taken from tail segments at 4 weeks of age identified 6 animals carrying the PEPCK *ada* gene (Fig. 2). A 2.3-kilobase DNA fragment hybridizing to the *ada* probe was identified after digestion with either *Xba*I or with *Sph*I and *Bam*HI. The 2.3-kilobase fragment is the expected size of tandem repeats of the PEPCK *ada trans* gene inserted in a head to tail fashion and should be isolated by *Xba*I digestion (see Fig. 1). The *Sph*I-*Bam*HI digestion should excise the intact *trans* gene of the same size. The presence of a 2.3-kilobase DNA fragment strongly hybridizing to *ada* indicates that the *trans* gene was inserted intact as head to tail concatemers. Other intensively hybridizing bands noted in Fig. 2 indicate that there are other more complex and unique integration patterns of the *trans* gene that also occur in the founder animals. In founders 2, 10, and 21, for instance, some copies of the *trans* gene appear to have lost adjacent *Bam*HI and *Xba*I restriction enzyme sites in the 5′ region of the *trans* gene, giving rise to hybridization of the *ada* probe to larger and identical DNA fragments in each instance when genomic DNA was digested with either *Xba*I or *Sph*I and *Bam*HI. In addition to these prominent bands, a faint band is seen in each lane of the group digested with *Xba*I which represents a "junctional fragment" between the *ada* gene and genomic DNA. If there is one insertion locus in each founder, one junctional fragment would be seen. This was, in fact, observed in DNA from each founder. Southern analysis of genomic DNA from the F_1 offspring of founders 10–49 digested with either *Xba*I or *Sph*I/*Bam*HI gave the identical pattern to that seen with the founder animals (data not shown). This also suggests that a single chromosomal insertion locus is present for each founder animal.

Table 1 shows the germ line transmission analysis for each of the founder animals. Founders 10 and 49 have relatively low numbers of gene copies per genome. These founders transmit the *trans* gene to approximately 50% of offspring as predicted if the gene is uniformly present in the germ line. Three founders, 14, 20, and 21, transmit with frequencies of 15–26% and are considered to have the *trans* gene present in a mosaic pattern in the germ line. Finally, founder 2, with more than 100 gene copies/genome, did not transmit the *trans* gene to any of his offspring.

Analysis of *ada* Gene Expression. To determine the level and tissue specificity of *ada* gene expression, F_1 offspring of the founder animals were killed, and total cellular RNA was pre-

Table 1 *PEPCK ada trans gene inheritance pattern by transgenic founders*

Six founder animals were analyzed for the number of gene copies/genome by Southern analysis of tail DNA. Outbred F_1 offspring were also analyzed for the transgene by slot blot of tail DNA. Transmission was characterized as heterozygote if approximately 50% of offspring carried the PEPCK *ada trans* gene and mosaic if 10–30% carried the transgene. The *ada* gene was used as probe.

Founder	Gene copies/ genome	F_1 transmission, positive/total (%)	Transmission
2 (M)	>100	0/29 (0)	None
10 (M)	5–8	22/38 (58)	Heterozygote
14 (M)	10–30	4/27 (15)	Mosaic
20 (M)	6–9	2/11 (18)	Mosaic
21 (F)	10–30	8/31 (26)	Mosaic
49 (M)	6–9	21/33 (64)	Heterozygote

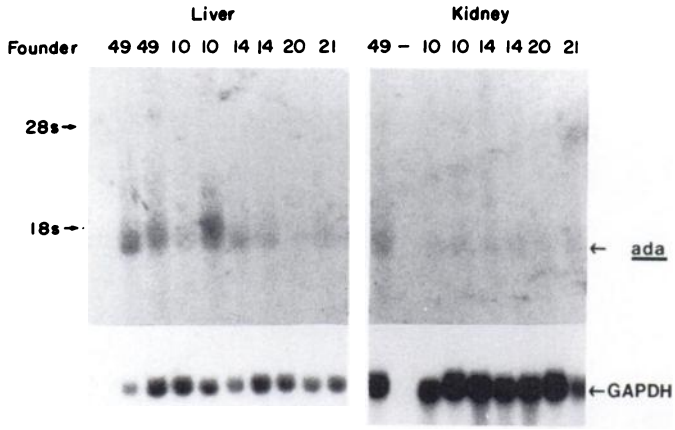

Fig. 3. *PEPCK ada* gene expression in liver and kidney of heterozygote offspring of founder animals. Twenty μg total cellular RNA, prepared from the liver and kidney of F_1 *ada*$^+$ heterozygote offspring of founder animals, was separated by formaldehyde-agarose gel electrophoresis and subjected to Northern analysis using the intact *ada* gene as probe. As a measure of the variability of mRNA loading in each lane, the membrane was also hybridized with the GAPDH probe as shown. Each lane represents a different animal and is labeled according to the founder parent. –, no mRNA loaded in the lane. A band representing the expected size of the *ada* mRNA transcribed from the PEPCK promoter is seen in each lane. No bands hybridizing with *ada* were seen in RNA isolated from nontransgenic mice (data not shown). The large sized hybridizing signal seen in the kidney of the founder 21 offspring may represent nonspecific hybridization with 28SR RNA and has not been seen in Northern analysis of other founder 21 offspring.

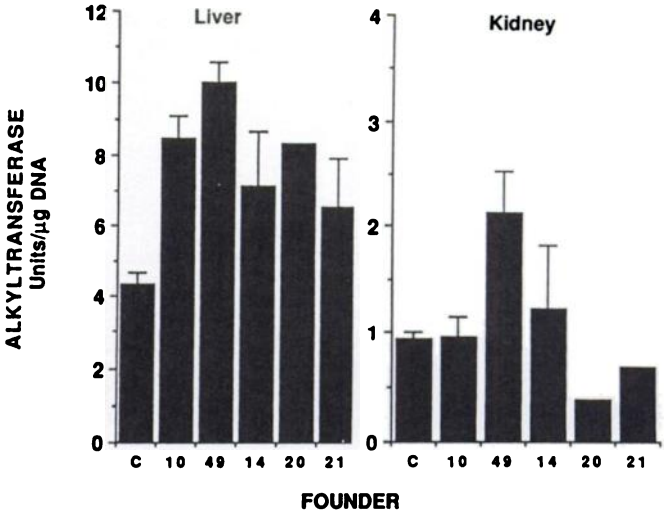

Fig. 4. Alkyltransferase in tissue of transgenic mice carrying PEPCK *ada*. Liver and kidney tissue extracts from nontransgenic control (*C*) and F_1 offspring of founder animals 10, 49, 14, 20, and 21 were assayed for alkyltransferase activity as described in "Materials and Methods." *Bar*, mean ± SD of the average of duplicate determinations of activity in 3–7 different animals except for founder 20 offspring in which only 2 animals were available.

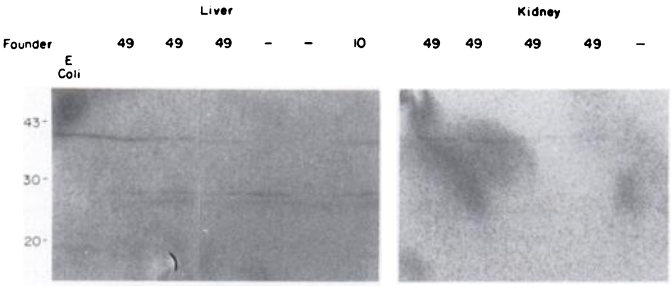

Fig. 5. Identification of bacterial alkyltransferase in transgenic mice. Cell extracts from liver and kidney containing 1 mg protein were reacted with 15 μg methyl-^{3}H DNA for 45 min at 37°C and the proteins separated by SDS-PAGE. An autoradiogram was exposed. The endogenous alkyltransferase has a molecular weight of approximately 23,000. A characteristic doublet or triplet (M_r = 22,000–26,000) was always observed in liver extracts. Alkyltransferase derived from the bacterial *ada* gene has a molecular weight of 39,000. *10* and *49*, founders from which F_1 offspring were derived; –, nontransgenic mice; *numbers on left*, M_r × 1000 of marker proteins.

pared from various tissues and analyzed by Northern blot using the 1.3-kilobase *ada* gene as probe. Fig. 3 shows analysis of RNA extracted from the liver and kidney from F_1 offspring of founders 10, 49, 14, 20, and 21. The amount of mRNA containing *ada* was much higher in the liver than kidney, whereas the level of endogenous PEPCK expression was similar in both tissues (data not shown). No RNA hybridizing with *ada* was observed in the lung or spleen (data not shown). There was some variation in the level of *ada* mRNA expression among F_1 offspring from different founders. However, all animals derived from the 5 founders expressed *ada* mRNA in the liver and kidney. The sex and age of the animals also did not seem to affect *ada* gene expression (data not shown).

Tissue Alkyltransferase Activity in Animals Carrying the PEPCK *ada trans* Gene. Fig. 4 shows the alkyltransferase activity in the liver and kidney in control animals (*C*) and outbred F_1 offspring from each of the transmitting founder animals. Alkyltransferase activity was measured as removal of O^6-methylguanine adducts from substrate [methyl-^{3}H]DNA, methylated with *N*-[^{3}H]nitroso-*N*-methylurea (32). Thus, activity in transgenic compared to control animals can be compared on the basis of relative capacity for removal of O^6-methylguanine DNA adducts. While the bacterial alkyltransferase will also remove O^6-methylthymine adducts and methylphosphotriester adducts (37), these are not measured in the enzyme assay we performed (34). Thus, a doubling in activity in transgenic *versus* control liver would indicate an equal number of alkyltransferase molecules of mammalian and bacterial origin. Each group of F_1 offspring had a 1.5–2.4-fold increase in alkyltransferase activity in the liver as compared to control animals. The levels of kidney alkyltransferase activity are much lower than in the liver, and F_1 offspring from founders 10, 14, 20, and 21 did not show increased renal alkyltransferase activity compared to nontransgenic litter mates. However, renal alkyltransferase activity was increased in offspring from founder 49; [2.1 ± 0.3 (mean ± SE) *versus* 0.9 ± 0.1 units/μg DNA]. Reduced renal alkyltransferase activity in founder 20 offspring may be an artifact of the small sample size (n = 2).

To confirm that the increased alkyltransferase was due to translation from *ada* mRNA, cell extracts were incubated with [methyl-^{3}H]DNA-containing O^6-[^{3}H]methylguanine DNA adducts to label functionally active alkyltransferase molecules of both bacterial and mammalian origin, and then the proteins were separated by SDS-PAGE. Fig. 5 shows that liver and kidney from transgenic animals contained a labeled protein of M_r 39,000, which was the expected size of the bacterial alkyltransferase protein transcribed from the *ada* mRNA. The bac-

terial alkyltransferase protein band was much stronger in liver extracts than in kidney extracts. Even though renal alkyltransferase activity was not increased in offspring of founder 10, a faint labeled protein of M_r 39,000 was seen, indicating that these animals also expressed the bacterial alkyltransferase in the kidney (data not shown). The amount of mammalian alkyltransferase found in the liver and kidney was the same in both transgenic animals and their nontransgenic litter mates. The molecular weight of mammalian alkyltransferase is about 23,000 (36) and multiple bands were always seen in the 22,000–26,000 molecular weight region in the liver, possibly due to the action of a nonspecific protease. In the kidney, the mammalian alkyltransferase was expressed at low levels and could only faintly be detected by SDS-PAGE.

Dietary Regulation of PEPCK *ada* Expression. The activity of hepatic PEPCK is induced by a diet high in protein but low in carbohydrate and markedly reduced by a diet high in carbohydrate (38). To test the regulation of the *trans* gene, F_1 offspring of founders 10 and 49 and nontransgenic litter mates were fed either regular rodent chow or a diet high in protein or high in carbohydrate for 1 or 4 weeks prior to being killed. There was a marked increase in *ada* mRNA in the liver of animals fed the high protein diet compared to transgenic animals fed a regular diet for 1 week (Fig. 6). We could detect no *ada* mRNA in animals fed the carbohydrate diet for the same period. Expression of the *ada* gene in the kidney was induced in 1 of 3 founder 10 offspring and 1 of 3 founder 49 offspring who were fed a diet high in protein (Fig. 7). However, PEPCK *ada* expression in the kidney was not reduced by a diet high in carbohydrate (data not shown). These results are consistent with the pattern of regulation of expression of the endogenous PEPCK gene in the liver and kidney (21, 24, 25, 39). Transgenic mice fed the high protein diet had a further increase in hepatic and renal alkyltransferase activity above that of nontransgenic (*ada*$^-$) and uninduced (*ada*$^+$) transgenic animals, whereas animals fed the carbohydrate diet had alkyltransferase activity in the liver and kidney that was similar to that seen in nontransgenic animals (Fig. 8). The induction of kidney alkyltransferase by a diet high in protein was greater in offspring of founder 49 than in offspring from the other founder animals. Hepatic alkyltransferase activity in transgenic animals fed a high protein diet for 1 week increased to 16.6 ± 1.5 units/μg DNA compared to 5.3 ± 0.6 units/μg DNA in the nontransgenic (*ada*$^-$) litter mates fed a high protein diet and 7.0 ± 0.8 units/μg DNA in carbohydrate-fed transgenic mice.

During these studies, we noted that there was no absolute correlation between *ada* mRNA levels and alkyltransferase activity, particularly comparing animals across founder lines. Within the same founder and the same tissue, there was a relatively good correlation between *ada* mRNA and alkyltransferase activity. Animals fed a high protein diet for up to 1 month showed similar increases in hepatic and renal alkyltrans-

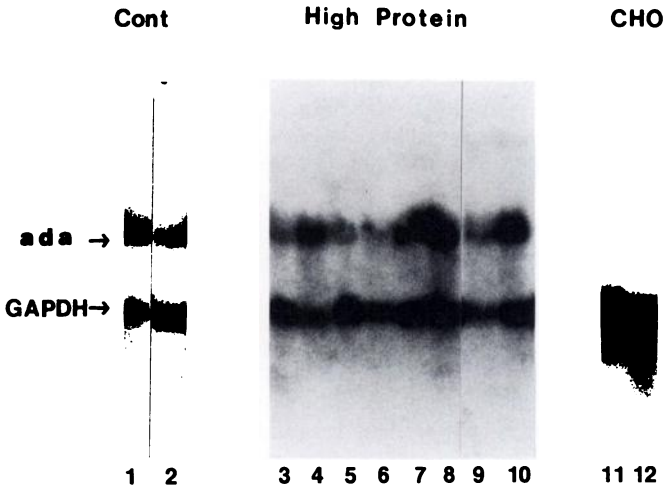

Fig. 6. Dietary regulation of PEPCK *ada* in the liver of transgenic animals. Transgenic mice were fed a regular diet (*Cont, Lanes 1* and *2*). A high protein diet (*High Protein, Lanes 3–10*) or a high carbohydrate diet (*CHO, Lanes 11* and *12*) for 7 days and 20 μg total cellular RNA extracted from liver was subjected to Northern analysis using the *ada* probe. The variability of mRNA loading is indicated by the intensity of the signal produced after probing the same membrane with GAPDH. GAPDH mRNA levels did not change during dietary manipulation. Increased *ada* expression was seen in liver from the animals on the high protein diet and decreased or absent expression was seen in animals on a carbohydrate diet. RNA isolated from offspring of founder 49 are shown in *Lanes 1–4* and *9–12* and from founder 10 in *Lanes 5–8*.

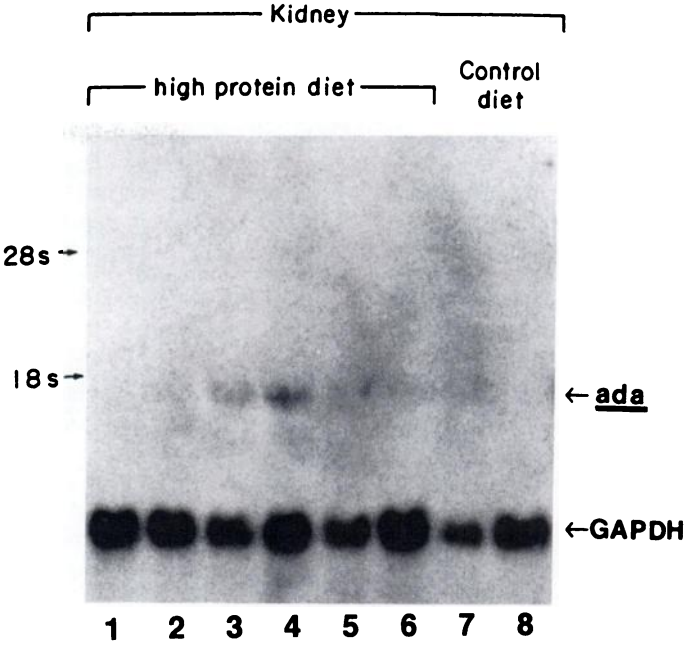

Fig. 7. Induction of PEPCK *ada* in kidney of transgenic animals fed a high protein diet. Transgenic animals were fed either a regular (control) or high protein diet for 7 days, and 20 μg total cellular RNA extracted from kidney was analyzed by Northern analysis using, sequentially, *ada* and GAPDH as probes. Increased expression of *ada* was seen in kidney of two animals fed the diet high in protein. *Lanes 1–3*, RNA from offspring of founder 10; *Lanes 4–8*, RNA from offspring of founder 49. The level of *ada* mRNA mice fed a control diet was greater in offspring of founder 49 than founder 10 (see Fig. 3).

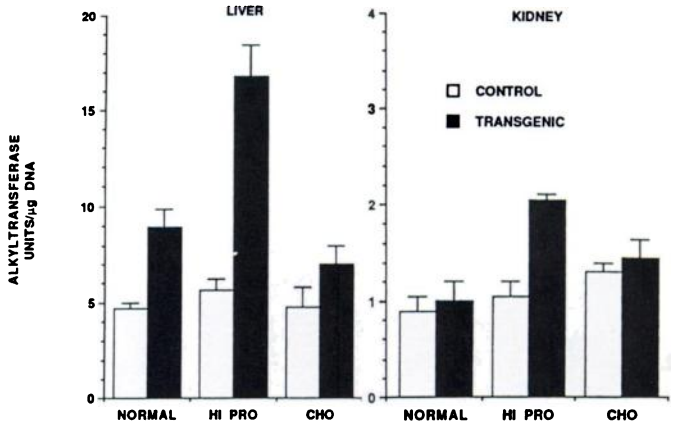

Fig. 8. Dietary regulation of liver and kidney alkyltransferase activity. Transgenic animals fed a regular (*NORMAL*), high protein (*HI PRO*), or high carbohydrate diet (*CHO*) for 7 days were sacrificed and liver and kidney were assayed for alkyltransferase. *White columns*, control nontransgenic *ada* litter mates; *black columns*, *ada*$^+$ transgenic mice offspring from founders 10 and 49. Data represent the means ± SE (*bars*)of duplicate determinations of the activity in tissues derived from 3–5 mice in each group.

ferase activity as that seen at 1 week. SDS-PAGE analysis of functional alkyltransferase (Fig. 9) shows that the increase in liver alkyltransferase in the transgenic animals fed a high protein diet was entirely due to bacterial alkyltransferase expressed in the mammalian tissues. Little or no bacterial alkyltransferase was detected in mice fed the diet high in carbohydrates. The resolution of the SDS-PAGE for kidney extracts was not sufficient to identify a change in alkyltransferase in animals fed a high protein diet (data not shown).

DISCUSSION

Our studies show that transgenic animals carrying the chimeric PEPCK *ada* gene have tissue-specific and -regulatable *ada* expression and appropriately increased alkyltransferase activity. These studies indicate that functional alkyltransferase of bacterial origin can be produced and expressed in transgenic animals. The transgenic animals we have characterized may provide an animal model for evaluating the impact of increased tissue alkyltransferase on *N*-nitroso toxicity in the liver and kidney. By targeting *ada* gene expression to liver and kidney, we have increased the alkyltransferase in these two tissues rather than in all tissues. The liver alkyltransferase activity obtained during induction with a high protein diet for 1 week (16.6 ± 1.5 units/μg DNA) is very high relative to that in other mouse tissues [range, 0.15–2.6 units/μg DNA (35, 39, 40)] and higher than that in rat tissues (range, 0–6.8 units/μg DNA) or human tissues [range 3.1–15 units/μg DNA (34, 35)] with the exception of human liver which has alkyltransferase activity of 55 ± 9.7 units/μg DNA (4, 34, 35). Thus, on a relative basis, the mouse liver of these transgenic animals should now be much more resistant to DNA damage induced by *N*-nitroso compounds than normal, nontransgenic mice of the same strain.

In the mouse, tissue alkyltransferase activity is lower than in other mammalian species such as human, rat, or monkey (35, 36, 40). This may be one reason that the mouse is susceptible to the toxicity and carcinogenicity of *N*-nitroso compounds (1, 2, 12, 41). It remains to be tested whether increased alkyltransferase activity will alter the toxicity profile in normal tissues of a number of *N*-nitroso compounds both in terms of acute cytotoxicity, DNA adduct formation, and overall carcinogenicity. A recent study has documented that expression of *ada* in a human tumor cell xenograft resulted in increased nitrosourea resistance (42). Their study was the first to document that expression of the bacterial *ada* gene will increase resistance to *N*-nitroso compounds in mammalian cells *in vivo*.

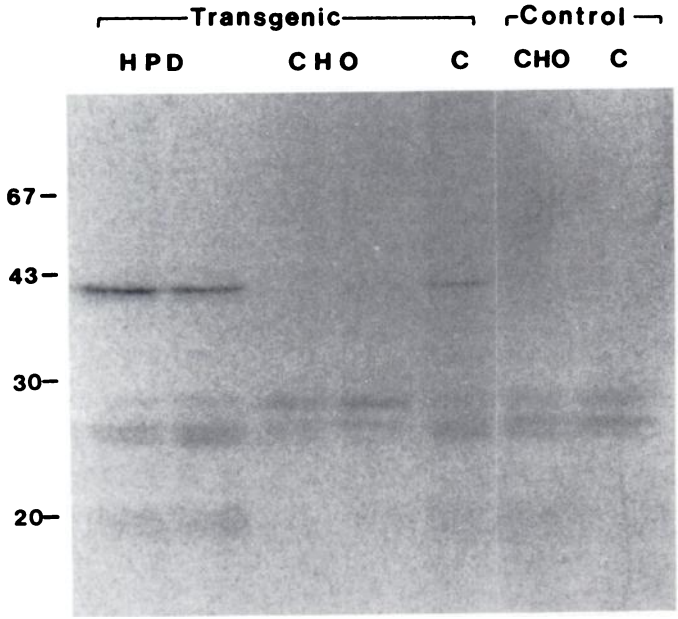

Fig. 9. Induction of bacterial alkyltransferase by a high protein diet in livers of transgenic animals. Liver tissue extract containing 1 mg protein was reacted with ^{3}H-DNA and separated by SDS-PAGE as described in Fig. 4. The increase in alkyltransferase activity seen in livers of animals fed a high protein diet was confined to the band of molecular weight 39,000 which is the size of the bacterial alkyltransferase protein coded for by *ada*. Note the absence of the protein of molecular weight 39,000 in the animals fed a high carbohydrate diet. *Transgenic*, offspring of founder 49; *control*, nontransgenic mice; *numbers on left*, $M_r \times 1{,}000$ marker proteins. *HPD*, high protein diet; *CHO*, high carbohydrate diet; *C*, control or regular diet.

Although we have measured removal of O^6-methylguanine from methylated DNA as an index of bacterial alkyltransferase expression, it should be noted that the bacterial protein differs from the mammalian alkyltransferase because it is also able to remove O^4-alkylthymine and alkylphosphotriester DNA adducts (43, 44). These adducts are formed by different *N*-nitroso compounds to varying extents relative to O^6-alkylguanine (10, 45), but the repair of these adducts by mammalian enzymes has not been extensively defined (43). However, the persistence of O^4-alkylthymine is associated with carcinogenicity (45, 46). Thus, it is possible that increased repair of these adducts, particularly O^4-alkylthymine, will influence carcinogenicity studies. Others have documented the breakdown of the intact bacterial alkyltransferase (M_r 39,000) to two functionally active fragments (M_r 19,000 and 20,000) in mammalian cells following transfection (17, 19, 47). This cleavage is thought to be due to a site sensitive to a thiol protease (48). While these cleaved fragments can function in mammalian cells, they were detected only in very low levels *in vivo* in our transgenic animals, indicating that most of the bacterial protein remains intact. If mammalian liver alkyltransferase sensitivity to a nonspecific protease is the explanation for the triplet band seen on SDS-PAGE (Figs. 5 and 8), it is of interest that this proposed protease does not affect the bacterial alkyltransferase. Addition of protease inhibitors does not alter total alkyltransferase activity/mg protein[5] but does seem to aid in the purification of the hepatic alkyltransferase (36).

The endogenous PEPCK gene is expressed in a highly regulated manner in the liver where it is inducible during gluconeogenesis and in the kidney where it is induced by acidosis (21–24). Previous studies with the chimeric PEPCK-bovine growth hormone gene in transgenic animals found the gene to be inducible in the liver by a high protein diet and inhibited by a diet high in carbohydrate (22). We have also found that the tissue specificity of PEPCK *ada* gene expression was retained when a truncated fragment of the 5′-flanking region of PEPCK was used. It is also possible that the 5th exon of the bovine growth hormone gene present in the PEPCK *ada trans* gene contributed to the stability of gene expression (for a review of factors affecting *trans* gene expression see Ref. 48).

While two other groups have published preliminary reports describing transgenic animals containing the metallothionein promoter linked to *ada* (50, 51), there are a number of advantages of the PEPCK promoter that make it useful in this system. Most importantly, the PEPCK promoter is inducible by dietary manipulations, whereas the metallothionein promoter requires the use of heavy metals, which are known to inhibit the alkyltransferase (52). Second, PEPCK *trans* genes can be turned off by a diet high in carbohydrate, whereas there is often a high level of basal expression from other promoters used in trans-

[5] S. L. Gerson, unpublished results.

genic animal work (49). In our studies of PEPCK *ada*, we found that neither the diet high in protein or carbohydrate altered endogenous mammalian alkyltransferase activity so that the change in the total alkyltransferase activity was due entirely to the bacterial protein. Third, McGrane *et al.* (53) have found that a PEPCK *trans* gene begins to be expressed at birth so that heterologous gene expression will not adversely affect animal development *in utero* (49, 54).

The ability to regulate expression of *ada* by transient dietary manipulation and thus to alter the liver alkyltransferase 3–4-fold may be important in the design of future carcinogenicity and DNA repair studies using *N*-nitroso compounds. Turning off expression of the bacterial alkyltransferase could be an important control in these experiments. Conversely, turning on the *ada* gene at various times after carcinogen exposure will help define the time course of DNA damage and repair as it relates to carcinogenicity. The use of O^6-methylguanine to deplete tissue alkyltransferase *in vivo* (55) is another possible modulator for these studies. Future carcinogenicity studies will be facilitated by the consistent pattern of PEPCK *ada* expression observed in the liver of F_1 offspring from multiple founder animals and in the kidney of founder 49 offspring. Understanding the role of a single DNA repair protein in tissue-specific carcinogenicity in these transgenic animals will help elucidate some of the basic mechanisms of tumor induction.

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