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# A Foreign Dihydrofolate Reductase Gene in Transgenic Mice Acts as a Dominant Mutation

JON W. GORDON

*Departments of Obstetrics, Gynecology and Reproductive Science and of Biochemistry, Mt. Sinai School of Medicine, 1 Gustave L. Levy Place, New York, New York 10029*

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**We have produced 17 lines of transgenic mice by microinjecting a full-length cDNA clone of an altered dihydrofolate reductase (*dhfr*) gene. The protein specified by this gene carries a point mutation which triples its $K_m$ for dihydrofolate and reduces substrate turnover 20-fold relative to the wild-type enzyme. Transgenic mice from different pedigrees, several of which carry a single copy of this gene in different integration sites, manifest an array of similar developmental abnormalities including growth stunting, reduced fertility, pigmentation changes, and skeletal defects. These defects appear in animals heterozygous for the foreign gene. RNA analyses demonstrate significant expression of the cDNA in newborn mice and adult tissues. These findings show that the additional *dhfr* gene exerts its mutational effects in a dominant fashion, and therefore the data indicate that transgenic mice can serve as models for elucidating mechanisms of dominant mutagenesis.**

The experimental insertion of retroviral (22) or molecularly cloned DNA into mouse embryos to produce transgenic mice (17) has rapidly led to informative studies of mammalian gene regulation (13, 16). In addition to their usefulness for expression studies, these gene transfer procedures can also produce phenotypically penetrant mutations accessible to study at the molecular level. When genes with important roles in development or cell differentiation are inactivated by the insertion of exogenous DNA, changes in the phenotypic characteristics of the animal can be used as markers for host gene disruption, and the injected gene can be used as a probe for molecular cloning of affected host sequences (21, 23, 37). Such phenotypic alterations usually appear as recessive mutations, except when the affected host gene functions in haploid cells (23, 24, 34, 49, 51). In the latter case, hemizygous mutations involving a single locus are analogous to homozygous recessive mutations in diploid cells. The mechanism by which these insertions lead to phenotypic change is apparent: all available copies of a host gene are disrupted by the integration event. These phenomena are comparable to spontaneous recessive mutations which occur in nature.

Dominant mutations, i.e., those in which changes at only one of two host loci lead to phenotypic alteration, are less well understood, and as yet gene transfer experiments have not produced a model representative of them. It is important to clarify how heterozygous changes at a single locus can in some cases profoundly affect the organism, while in most instances such heterozygotes are phenotypically normal. One can envision the creation of dominant mutations by gene transfer in either of two ways: insertional disruption of a host gene, both copies of which are necessary for maintenance of a normal phenotype, or insertion of altered genes which, when expressed, function as dominant mutant alleles of host genes.

We have produced a series of transgenic mice which appear to provide an experimental model of dominant mutagenesis based on the latter of these two mechanisms. We produced these animals by microinjecting a full-length expressible cDNA of an altered dihydrofolate reductase (*dhfr*) gene. This gene, originally identified in cultured mouse 3T6 cells, has a point mutation in the 5′ region which raises the $K_m$ of the resulting enzyme threefold and reduces dihydrofolate turnover by a factor of 20 (19). The full-length cDNA was inserted into an expression vector with a promoter from simian virus 40 (SV40) and a polyadenylation sequence from human hepatitis B virus (HBV) (39). The construct had previously been shown to function efficiently after transfection into cultured cells (39). Several of the transgenic mice carried a single copy of the new gene as a heterozygous trait. These animals manifested a variety of developmental defects, while control littermates in all lines were normal. RNA hybridization studies provide evidence that the new gene's dominant mutational effects result from expression. In this report the abnormalities found in our transgenic mice are described, and a mechanism whereby heterozygous changes in the folate reductase pathway can cause developmental abnormalities is proposed.

## MATERIALS AND METHODS

**Production of transgenic mice.** The recombinant plasmid pFR400 (39), the restriction map of which is shown in Fig. 1, was prepared for microinjection in two ways. In one set of experiments, a 2,825-base-pair (bp) fragment produced by digestion with *Sal*I and *Pst*I and carrying all sequences needed for expression was electroeluted from agarose and injected. The second group of injections was performed after linearization of the 4,400-bp plasmid with *Pst*I.

CD-1 mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass. B6D2F/1 mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. Zygotes were obtained for microinjection by superovulation of immature female CD-1 animals followed by mating to B6D2F/1 males. Recovery of fertilized eggs and microinjection of pronuclei was performed as previously described (14, 15, 17). The most accessible pronucleus, usually the male, was microinjected with 200 to 2,000 copies of the linearized plasmid.

**DNA isolation and hybridization analysis.** For initial screening, high-molecular-weight DNA was prepared from

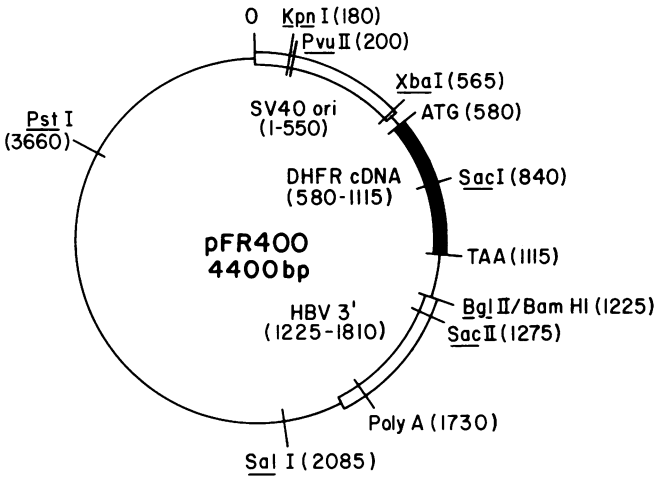


FIG. 1. Restriction map of pFR400 (39). The thin line denotes plasmid sequences. Open bars indicate the SV40 promoter (0 to 550) and the HBV polyadenylation sequence (1225 to 1810). The solid bar indicates the DHFR cDNA (580 to 1115). The *Sal*I site at position 2085 and the *Pst*I site at 3660 were those digested prior to microinjection.

spleen biopsies by the method of Blin and Stafford (1), with modifications as described previously (17). DNA (10 μg) was then digested to completion with *Xba*I, blotted onto nitrocellulose (41), and hybridized to nick-translated pFR400. Nick translations were performed with kits obtained from New England Nuclear Corp., Boston, Mass., and conducted to yield a probe with $10^8$-dpm/μg incorporation of [$^{32}$P]dCTP. Filter hybridizations were performed by the method of Wahl et al. (50). All lineages wherein a single copy of pFR400 was integrated were studied by Southern blot analysis. When multiple copies of the plasmid were integrated, transmission of the foreign gene was monitored by dot blot analyses of tail DNA.

For tail blot analyses, the distal third of the tail was removed from weanling mice. The skin was minced and subjected to DNA extraction by the method of Davis et al. (10) as modified by Stewart et al. (42). Of the tail DNA, 1 μg was denatured and applied to nitrocellulose for hybridization analysis. Hybridizations were performed in the same manner as Southern blot hybridizations.

For animals presumed to carry single-copy inserts of pFR400, all Southern analyses included a positive control made by digesting pFR400 with *Sal*I and *Pst*I and loading a slot with amounts equivalent to one gene copy per cell. Southern analyses of mice carrying multiple copies of exogenous DNA included a lane containing pFR400 digested with *Pst*I and loaded in amounts equivalent to four copies per cell. *Pst*I-digested pFR400 was also loaded in amounts equivalent to 10 copies per cell on dot blots.

**RNA isolation and analysis.** RNA was isolated from liver and whole newborn animals by a modification of the method of Glisin et al. (12). Up to 0.5 g of tissue was placed in 2.5 ml of extraction buffer containing 4 M guanidinium isothiocyanate, 1 M sodium citrate added to a final concentration of 5 mM, 0.1 M 2-mercaptoethanol, and 0.5% *N*-lauryl sarcosine. The homogenate was layered on a 1.2-ml cushion of 5.7 M CsCl in 0.1 M EDTA (pH 7.5) and spun at 35,000 rpm in a Beckman SW55 Ti rotor for 12 h. Supernatants were discarded, and pellets were redissolved in 0.5 ml of 10 mM Tris hydrochloride (pH 7.4)–5 mM EDTA– 1% sodium dodecyl sulfate. Dissolved pellets were extracted with 3:1 $CHCl_3$-butanol, and RNA as precipitated by the addition of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol.

For dot blot analyses, 30 μg of RNA was denatured in 100 μl of 50% formamide at 65°C for 10 min. After denaturation, the RNA was washed in 20 μl of 6× SSC (20× SSC is 3 M NaCl–0.3 M sodium citrate [pH 7.0]). RNA was then applied to GeneScreen hybridization membranes (New England Nuclear) by a dot blotting device. For Northern analyses, RNA was denatured in glyoxal-dimethyl sulfoxide, electrophoresed in 1.5% agarose dissolved in 0.1 M $NaPO_4$ (pH 7.0), stained with ethidium bromide, and blotted onto GeneScreen hybridization membranes with 20× SSC. RNA size was estimated with 20S and 18S rRNA bands as standards.

Solution hybridizations for both dot blots and Northern blots were performed by the method of Pintar et al. (35). In dot blot hybridizations, two probes were used; a 1,520-bp fragment extending from the *Xba*I site to the *Sal*I site and homologous both to pFR400 and to the endogenous *dhfr* transcript was electroeluted and nick translated. In other experiments, the 810-bp *Sac*II-*Sal*I fragment containing HBV polyadenylation sequences and therefore specific for the injected gene (Fig. 1) was used as a probe.

## RESULTS

**Production of 17 lines of transgenic mice, with 4 lines containing single copies of pFR400.** Table 1 summarizes the microinjection results and shows that 5 animals were produced in the first series and 12 were produced in the second. Mice injected with the 2,825-bp *Sal*I-*Pst*I fragment were designated 401 through 436; those receiving the *Pst*I-linearized material were numbered P401 through P450. Transgenic mouse P439 died before it could be analyzed further. Thus, 17 distinct lineages, 16 of which were available for study, were produced.

To evaluate the state of the integrated material in transgenic animals, a simple restriction enzyme analysis was undertaken. DNA samples were digested with *Xba*I, an enzyme which recognizes a single site within the SV40 region of the plasmid (Fig. 1). Integration of a single copy of the donor sequence would be likely to produce *Xba*I bands different in size from the injected unit, as *Xba*I digestion would generate fragments composed in part of the plasmid and in part of mouse genomic DNA. These fragments would hybridize to a radiolabeled probe with equal or slightly greater intensity than the host gene. In contrast, *Xba*I digestion of head-to-tail concatamers would yield bands of high intensity with the same mobility as the injected sequence. Figures 2 and 3 show results of this analysis from animals injected with the *Sal*I-*Pst*I fragment (Fig. 2) and the 4,400-bp *Pst*I fragment (Fig. 3) of pFR400. Four of the five animals identified from the first series—mice 406, 407, 414,

TABLE 1. Results of microinjection[a] of two constructs of pFR400

| Fragment (size) injected | No. of embryos | | No. of mice | |
|---|---|---|---|---|
| | Injected | Implanted | Born | Positive (%) |
| *Sal* I-*Pst* (2,825 bp) | 587 | 382 | 36 | 5 (13.8) |
| *Pst*I (4,400 bp) | 573 | 413 | 50 | 12 (24.0) |

[a] The most accessible pronucleus of fertilized eggs was microinjected with 200 to 2,000 copies of a plasmid derivative.

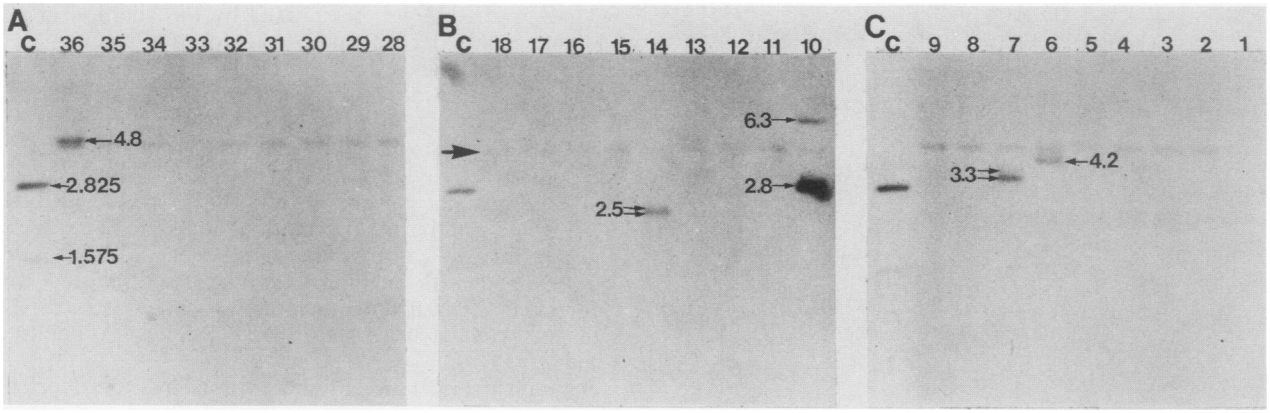


FIG. 2. Southern blot hybridizations of *Xba*I-digested, pFR400-probed spleen DNAs from mice 401 through 418 and 428 through 436. These animals were microinjected with the 2,825-bp derivative of pFR400 digested with *Sal*I and *Pst*I. The control (lanes c) consisted of pFR400 digested with *Sal*I and *Pst*I and loaded in amounts equivalent to one copy per cell (10 pg). The exact sizes of the control fragments are shown in panel A. Positions of restriction fragments derived from pFR400, identified in mice 406, 407, 410, 414, and 436, are indicated by arrows. Fragment sizes were estimated by using known values for the positive control and for lambda DNA digested with *Hin*dIII and visualized after ethidium bromide staining. Double arrows in samples from mouse 407 and mouse 414 indicate the presence of doublet bands. A prominent fragment of the endogenous gene, indicated by the large arrow, is present in all transgenic mouse samples and is identical in intensity and position to those of negative mice. This band is not visible in sample 433, owing to incomplete digestion of the DNA.

and 436—retained a single copy of pFR400 per cell. Mouse 410 carried a short concatamer determined by dilution studies to contain 5 to 10 plasmid copies. Figure 2 also shows several negative animals from the initial screening procedure. Only fragments of the endogenous gene can be seen in these cases. All 12 animals retaining the 4,400-bp *Pst*I fragment harbored head-to-tail concatamers (Fig. 3). Retention of concatamers was demonstrated by the presence of intense 4,400-bp bands in all lanes. Fragments of lower intensity indicative of the unique structure of each insert, are also visible in several samples.

The unique sizes of the *Xba*I fragments in all single-copy integrants show that the relative positions of genomic *Xba*I sites to the integrated plasmid are different in each instance and thus that the integration site is unique in each pedigree. Also visible in all positive and negative samples is a fragment of the endogenous *dhfr* gene. The intensity and position of this band is the same in samples from both transgenic mice and normal animals. These data thus indicate integration of a single copy of the donor plasmid into a distinct site in the genome in four of five cases.

It should be emphasized that the mouse *dhfr* gene is quite large and contains many introns which would not hybridize to our cDNA probe (9). The endogenous gene could thus

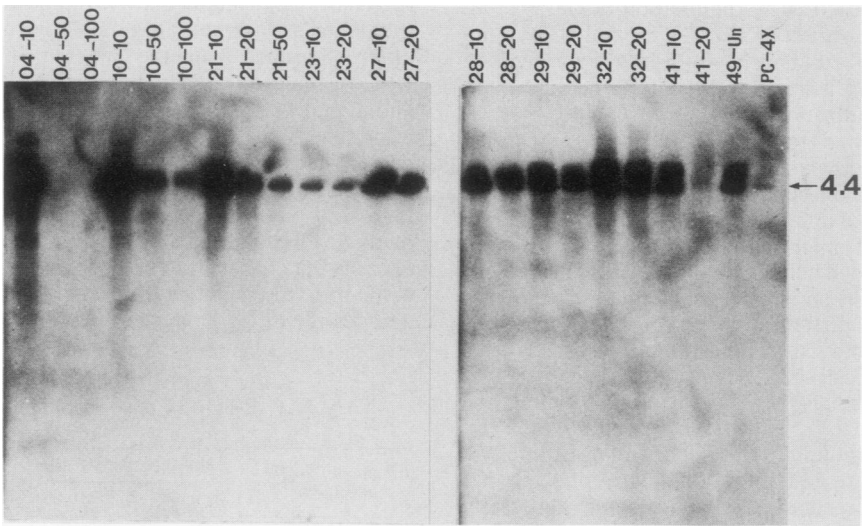


FIG. 3. Southern blot hybridization of *Xba*I-digested spleen DNA from several transgenic mice injected with the 4,400-bp *Pst*I fragment of pFR400. Blots were probed with pFR400. The number of the lineage is shown, with the degree of DNA dilution prior to digestion shown after the hyphen in each sample. Line P447, estimated after initial screening to carry 5 to 10 copies per cell, is not included. Line P449 DNA was undiluted prior to digestions. A positive control composed of pFR400 digested with *Xba*I and loaded in amounts equivalent to four copies per cell is shown as PC-4X. The 50- and 100-fold dilutions of P404 DNA and the 20-fold dilution of P441 DNA were degraded. The 4.4-kilobase linearized control plasmid is indicated by the arrow.

TABLE 2. Progeny testing of pFR400 transgenic mice

| Mouse no. | Sex | Avg litter size (no. of litters scored)[a] | No. (%) of positive mice | |
|---|---|---|---|---|
| | | | Generation 1 | Generation 2 |
| 406 | M | 7.0 (2) | 4/13 (31) | 5/8 (63) |
| 407 | F | 4.3 (4) | 3/9 (33) | 19/35 (54) |
| 410 | F | 4.5 (6) | 7/13 (54) | 13/27 (48) |
| 414 | M | 3.3 (5) | 6/20 (30) | 20/49 (41) |
| 436 | F | 6.5 (2) | 9/13 (69) | NT[b] (NT) |
| P404 | F | 0.6 (5)[c] | 1/3 (33) | —[d] (—) |
| P410 | F | 8.4 (5) | 22/39 (56) | NT (NT) |
| P421 | M | 0 (5) | 14/47 (30) | NT (NT) |
| P423 | M | 11.0 (1) | 23/46 (50) | 4/11 (36) |
| P427 | M | NT | 12/32 (38) | NT (NT) |
| P428 | M | NT | 22/37 (59) | NT (NT) |
| P429 | F | 8.0 (3) | 13/24 (54) | 7/7 (100) |
| P432 | M | 7.5 (2) | 10/59 (12) | 4/7 (57)[e] |
| P441 | M | 13.0 (1) | 21/74 (28) | NT (NT) |
| P447 | F | 8.0 (2) | 7/16 (44) | NT (NT) |
| P449 | F | 11.0 (6) | 10/24 (42) | NT (NT) |

[a] The number of animals scored for litter size is occasionally less than the number screened by Southern hybridization for pFR400 DNA because some of the animals born have not been screened as yet. Females were not considered to have a reduced litter size unless an average of less than six offspring was recorded.
[b] NT, Not tested.
[c] Four documented matings with no births.
[d] —, No successful matings subsequent to the first generation.
[e] Two heterozygotes were crossed.

sustain integration of pFR400 at many positions without causing a change in the Southern hybridization pattern observed in our experiments. Moreover, in animals carrying multiple copies of pFR400, characteristic junction fragments establishing a unique integration site in each pedigree could not always be visualized (Fig. 3). However, in view of the size of the mouse genome and the extensive evidence indicating random integration of foreign DNA in transgenic mice (8, 17, 26, 31), we consider it unlikely that either host *dhfr* allele is affected in any of the animals. In any case, as genetic studies confirm single, independently segregating integration sites for pFR400, no more than one host *dhfr* allele could possibly be interrupted in any pedigree.

The conclusion that the first four animals carried single copies of the foreign gene could be spurious if modified concatamers were integrated in a mosaic distribution in founder mice. Several laboratories have shown that foreign-gene integration may take place in only some cells of the embryo, an occurrence which leads to a mosaic adult animal (8, 31, 34, 43). We have ruled out this possibility by breeding the animals (Table 2). In all four cases, the transmission frequency of pFR400 was not significantly lower than 50%, as would be expected from a mosaic parent. Moreover the hybridization intensity in offspring was not greater than in the parents, which would be predicted if the parents were mosaics.

**Foreign *dhfr* gene acts as a dominant mutation.** The presence of this alien *dhfr* gene as a heterozygous trait in the transgenic mice is associated with a variety of developmental anomalies. The abnormal traits are heritable and invariably linked to pFR400. Examinations of the transgenic mice have revealed stunted growth (11 lines), reduced fertility (5 lines), skeletal (10 lines) and eye (3 lines) deformities, and pigmentation defects (2 lines) (Table 3). None of the negative littermates was abnormal. Examination of more than 1,000 mice of similar genetic background, housed in the same room and derived from transgenic mice microinjected with other DNA sequences, has likewise revealed none of the anomalies characteristic of these transgenic animals. Several of the normal transgenic lineages in the laboratory carry the regions of both SV40 and HBV DNA contained in pFR400, suggesting that these sequences are not responsible for the effects of pFR400 integration.

An obvious anomaly encountered in several lines was stunting of growth. Several founder mice, particularly 410 and P404, were noted to be smaller than their littermates when initially screened for pFR400 sequences. In the case of mouse P404, the reduced size was especially remarkable. When weaned, this animal weighed less than 8 g, while its negative littermates weighed more than 25 g. Mouse P404 never attained a weight of greater than 17.8 g, i.e., less than half that of its normal sibs. This female completed one successful pregnancy and delivered three young. One pup was very small at birth, while the others appeared normal. The offspring were monitored with growth curves (Fig. 4). Two mice grew normally, while the third remained very small. All three offspring were screened at 30 days of age by dot blot hybridization of DNA obtained from tail biopsies. This test confirmed that the small mouse was a carrier of pFR400 sequences, while the normal-sized pups were not. Growth of the transgenic pup was markedly retarded. This animal was maintained for 22 weeks and achieved a maximum weight of 3.7 g, approximately 1/10 that of a normal age-matched mouse. Various degrees of growth stunting at birth were seen in several additional lines (Table 3). In dwarf transgenic mice, the rate of maturation was not changed. As a result, adult mice no larger than 5-day-old pups were frequently seen (Fig. 5A).

Another obvious abnormality was spinal deformity, manifested as kinking of the tail. This effect was most pronounced in line P441 (Fig. 5B). However, several other lines showed unmistakable tail anomalies. A single kink at the end of the tail was seen in line 414, while animals from the P410 lineage had two kinks, one in the middle region and one at the end. Lumbar deformities in P410 and several other

TABLE 3. Summary of abnormalities in transgenic mice carrying pFR400[a]

| Mouse no. | Gene copies per cell | Abnormal tails | Stunted growth | Eye abnormalities | Reduced fertility[b] | Pigmentation defects |
|---|---|---|---|---|---|---|
| 406 | 1 | + | + | − | − | − |
| 407 | 1 | + | + | − | + | + |
| 410 | 5–10 | − | + | + | +* | − |
| 414 | 1 | + | + | − | +* | + |
| 436 | 1 | + | − | − | − | − |
| P404 | >400 | + | + | − | +* | − |
| P410 | >400 | + | + | + | − | − |
| P421 | 200–400 | + | + | − | + | − |
| P423 | 200 | + | + | − | − | − |
| P427 | 200 | − | − | − | − | − |
| P428 | 200–400 | − | − | − | − | − |
| P429 | 200–400 | + | − | − | − | − |
| P432 | 200–400 | − | + | + | * | − |
| P441 | 200 | + | + | − | − | − |
| P447 | 5–10 | − | + | − | − | − |
| P449 | 100 | − | − | − | − | − |

[a] No obvious association exists between any two of the defects, and no correlation exists between the number of gene copies per cell and the variety or severity of problems seen. +, Mouse showed defect; −, mouse did not show defect.
[b] +, Reduced litter size; +*, late onset of fertility.

pedigrees suggest that skeletal defects involve proximal regions of the spine as well.

Pigment cell defects were seen in the 407 and 414 lineages. Here, patches of white hair are prominent (Fig. 5C). In line 407 mice, a white patch was evident at birth, while line 414 mice developed white fur postnatally. In the 407 lineage, the patch was stable throughout adult life, while white areas progressively enlarged in line 414 mice.

In lineages 410, P410, and 432, animals were born with their eyes open (Fig. 5D). This anomaly, most severe in the 410 lineage, often led to corneal opacity and blindness in adults.

Other transgenic animals had reduced fertility, discovered by screening 628 progeny from first- and second-generation crosses in several lines (Table 2). The abnormality took two forms: delayed onset of fertility and reduced litter size of females. In lines 407, 410, 414, P404, and P431, subfertility was clearly evidenced by reduced litter size of female transgenic mice (Table 2). We investigated the possibility that transgenic embryos might fail to survive until birth. If this were the case, we would expect them to constitute fewer than 50% of progeny. The data show that pFR400 was transmitted to about 50% of animals in all but two lines (Table 2). In lines P441 and P432, germ line mosaicism was present in the founder mice. However, transmission of pFR400 in second-generation crosses was approximately 50%. It is possible that in line 414 mice some death of transgenic conceptuses was taking place. Testing of 69 progeny over several generations in this line demonstrated an overall transmission frequency of 38% (Table 2).

Mouse P404 passed the new gene to one of three pups in its single litter. In this instance we have the strongest evidence of abnormal fetal loss. When it failed to produce further litters after five documented matings, one after superovulation, the animal was killed at 40 weeks of age. Examination of the uterus revealed one morphologically normal full-term fetus, which proved negative for pFR400 after Southern blotting, and two resorption sites. Nucleic acid extraction of the resorption tissues was not sufficient to separate embryonic from contaminating maternal DNA. The parent's ovaries were removed and studied histologically. The number of germ cells per unit area was similar to that of normal mice. The appearance of the dead fetus and the resorption sites at the time of sacrifice suggests that some normal animals could not be delivered because of the small size of the birth canal in the parent and that others, presumably the transgenic embryos, died shortly after implantation.

Females of the P421 lineage were infertile. After five documented matings of three females with two normal males, no mice were born (Table 2). After a fifth mating, one female was examined surgically for implantation sites. Two normal conceptuses and no resorption sites were observed. To determine if normal oocytes were present, a female was superovulated and sacrificed the day of mating. Of 24 oocytes recovered, 13 were fertilized. To evaluate the developmental potential of fertilized eggs from this line, another female and a negative control from the same pedigree were superovulated, mated, and sacrificed 49 h after administration of human chorionic gonadotropin (Table 4). At that time, conceptuses would be expected to have reached the 8-cell (morula) stage of development. The oviducts were flushed, and the in vitro development of the embryos within them were evaluated in comparison with those of a littermate which did not carry pFR400 (Table 4). Only 3 of 19 embryos recovered were at the morula stage and normal in appearance. After an additional 24 h in culture, when 15 of 18 controls had reached the blastocyst stage, only two embryos from the transgenic mouse were blastocysts. However, after another 24 h of culture, 17 of the embryos were blastocysts. These findings indicate that the rate of cleavage is slowed in P421 embryos and that the paucity of successful implantation events may be due to discordant timing of uterine maturation and embryonic development.

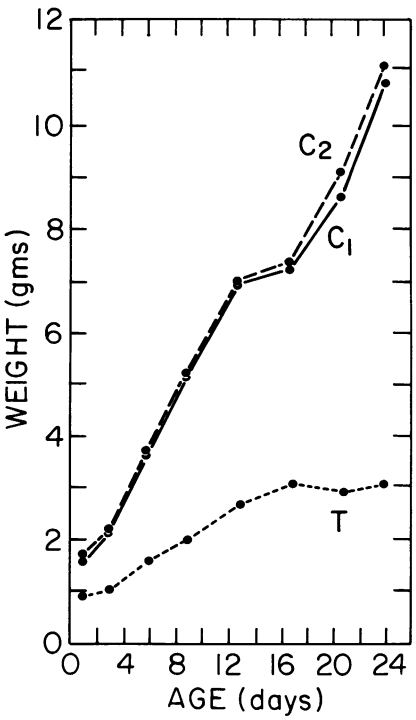


FIG. 4. A growth curve obtained from three pups born to transgenic mouse P404. Weight in grams was plotted against age for the first 24 days of postnatal development. The animals were subsequently screened for pFR400 sequences by dot blot hybridization of tail DNA. Tail blots of these mice showed that the small animal was a transgenic mouse and the other two were normal (see text).

**Abnormal phenotypes are of similar character within lines but have variable penetrance.** Within each pedigree that manifested abnormalities, the nature of the abnormality was remarkably consistent. Thus, all line 414 transgenic mice with tail kinks showed a similar pattern of kinking, i.e., a small kink at the end of the tail. The consistency of appearance of the developmental defects was most easily seen in the spotted mice of the 407 line. Shortly after birth, when these animals began to develop pigment, a white area appeared on the dorsal midline of the lumbar spine. The reproducibility of the location in which the white spot appeared is demonstrated in Fig. 5C, which depicts seven transgenic mice from this line. The same region of the lumbar spine is affected in each case. Different-sized spots reflect variable penetrance of the developmental defect. The phenomenon of variable penetrance was observed for all anomalies identified and may be related to the mixed genetic background of the animals.

**Developmental abnormalities are associated with pFR400 expression.** The presence of developmental defects in transgenic mice heterozygous for pFR400 and the similar character of the abnormalities in lines carrying the donor gene in different genomic locations suggested that pFR400 expression was responsible for our observations. We there-

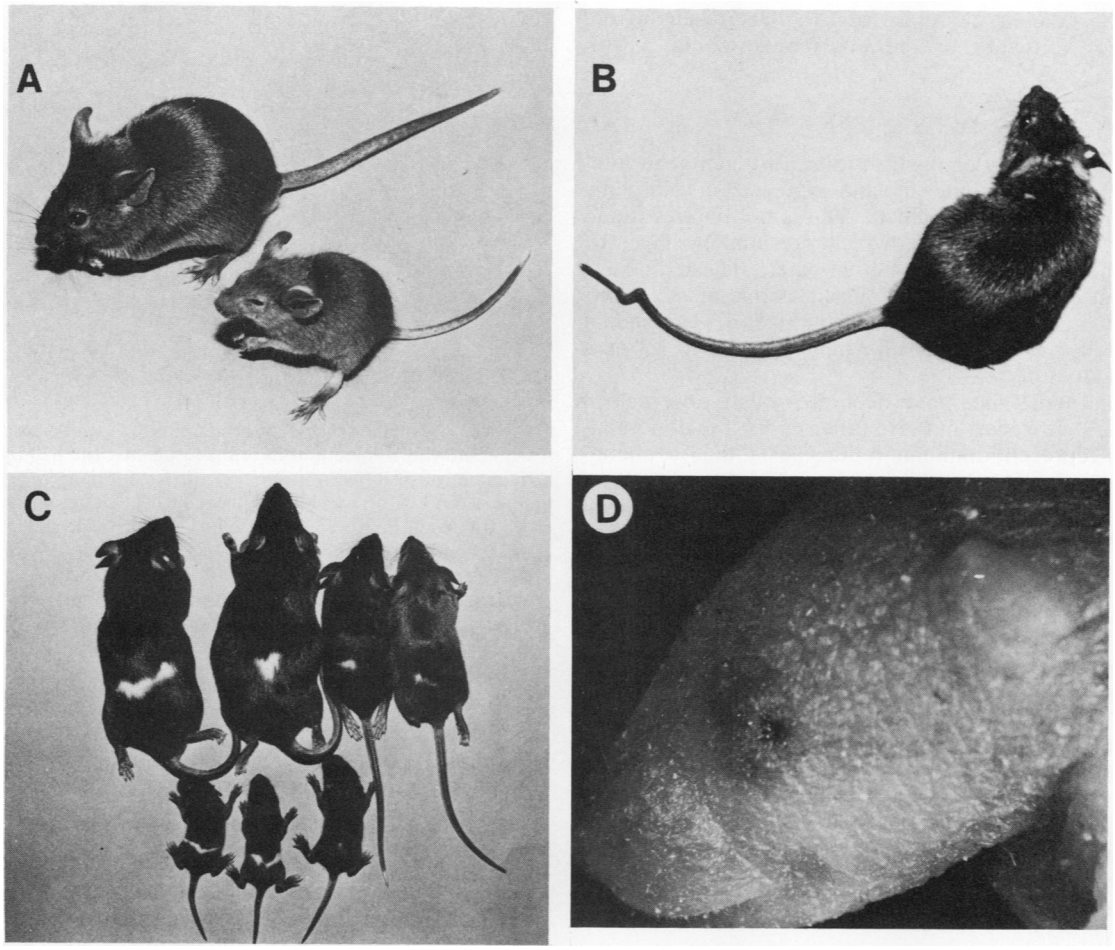


FIG. 5. Abnormal transgenic mice which carry pFR400 sequences. (A) A transgenic mouse from the 410 line, foreground, with a normal littermate of the same sex. Stunted growth in the transgenic animals is pronounced. (B) Transgenic mouse P441, showing a markedly kinked tail. (C) Seven transgenic mice from three generations of the 407 line, showing variable loss of fur pigmentation in the dorsal lumbar region. (D) A newborn animal from the P432 pedigree. The eye is open.

fore analyzed several transgenic mice for transcription of the *dhfr* gene. Prior to conducting these experiments, we recognized the problem that developmental anomalies seen in newborns, if based on pFR400 expression, undoubtedly result from gene function earlier in development. The absence of expression in newborns or adults therefore does not negate the possibility of significant expression early in ontogeny.

With these issues in mind, we initiated expression studies with a dot blot assay designed to test for increased *dhfr* gene expression. The entire pFR400 plasmid was used as a probe against RNAs from several newborn transgenic mice, normal controls, and adult liver. Because the ability of foreign genes to express in transgenic mice depends in part on the integration site of the gene (2, 6, 25, 26, 31), we reasoned that this test, although conducted at arbitrarily chosen stages of development, would at least determine if the foreign gene was integrated into a site permissive for expression. The results of these experiments (Fig. 6a) clearly demonstrate increased *dhfr* gene transcription in both newborns and adult livers in transgenic animals from lines in which developmental anomalies were observed. We then exposed the same samples to hybridization with a purified HBV polyadenylation sequence probe produced by nick translation of the 810-bp *Sac*II-*Sal*I fragment of pFR400 (Fig. 1). Figure 6b shows specific hybridization of this fragment to RNA from transgenic mice, with minimal hybridization to control samples. Although the signal was not strong, follow-up Northern blot hybridizations from additional samples showed a single prominent RNA species of about 1.2 kilobases which hybridized to the purified HBV prove (Fig. 7). This is the size expected from appropriate transcription of pFR400 followed by polyadenylation at the HBV site (Fig. 1). We thus conclude that a vast majority, probably all pFR400 transcripts, are polyadenylated at the HBV site. These experiments demonstrate transcriptional activity of the foreign gene and thus correlate pFR400 transcription with the developmental defects seen. The data show no correlation

TABLE 4. Cleavage rate in vitro of embryos from the P421 line

| Time post-HCG[a] (h) | No. of transgenic mice showing: | | | No. of control mice showing: | | |
|---|---|---|---|---|---|---|
| | 4 cells | Morula | Blastocyst | 4 cells | Morula | Blastocyst |
| 49 | 16 | 3 | 0 | 4 | 14 | 0 |
| 73 | | 17 | 2 | 1 | 2 | 15 |
| 97 | | 2 | 17 | 1 | 1 | 16 |

[a] HCG, Human chorionic gonadotropin.

between the number of copies of pFR400 present in the animals and the level of expression (compare Fig. 6 with Table 3).

## DISCUSSION

The data collected demonstrate that a foreign gene integrated into a single genomic site and expressed in transgenic mice can have mutagenic effects. The abnormalities found are similar in separate lines, which presumably have the foreign gene integrated at different sites. These findings indicate that even though it is present as one of three *dhfr* alleles in the mice, the new sequence induces developmental lesions. Thus, the additional *dhfr* gene appears to act as a dominant mutation.

Other transgenic mice have been previously observed to be abnormal. However, in these other cases, the abnormalities do not represent dominant mutagenesis, that is, phenotypic change despite the existence within the animals of normal alleles of the inserted sequences. SV40 T-antigen expression in transgenic mice causes a variety of abnormalities (2, 29, 32, 40), but this product is entirely foreign to the animal and has no normal counterpart. Animals secreting foreign immunoglobulins experience allelic exclusion of their host loci and thus are functionally hemizygous for the foreign gene (36). Animals unable to transmit foreign genes through the male germ line have transmission distortion mosaicism, apparently as a result of insertional mutagenesis within a host gene which functions postmeiotically. Therefore, the affected cells are once again functionally hemizygous (34). A number of genes cloned from heterologous species, including the herpesvirus thymidine kinase gene, the chicken transferrin gene, the rat elastase I gene, and the human globin and *hprt* genes, have been inserted and efficiently expressed in transgenic mice (3, 28, 31, 45, 46, 48). Because of their structural differences from the corresponding mouse proteins, any of these genes might serve as mutant "alleles" in mice. However, in none of these cases were the transgenic animals phenotypically abnormal. Several differences between these other experiments and those reported here might explain why the mutant *dhfr* gene acts as a dominant mutant allele. In the aforementioned cases, embryonic expression may not have taken place. Moreover, many of the other foreign proteins are catalytically similar to their mouse counterparts.

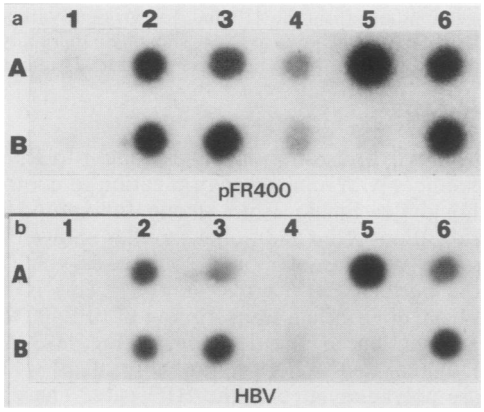


FIG. 6. Dot blot hybridization of RNA obtained from newborn mice (rows A) and adult livers (rows B) from several transgenic mouse pedigrees. Samples A1 and B1 are negative controls. Experimental samples were loaded with the following lines: A2, 407; A3, 414; A4, P410; A5, P421; A6, P428; B2, 436; B3, P404; B4, 406; B5, P429; and B6, P432. Panel a shows samples hybridized to nick-translated pFR400. In panel b, the same samples were hybridized to the purified 810-bp *Sac*II-*Sal*I fragment of pFR400 which carries only HBV poly(A) sequences. Samples A2, A3, B2, and B4 show that all single-copy carriers expressed pFR400. Sample B3 shows markedly elevated pFR400 transcription in this severely stunted transgenic mouse. Sample A4 shows detectable but relatively low transcription in a severely abnormal newborn pup. Comparison of B2, a sample obtained from a single-copy animal, and B5, obtained from a mouse harboring a large concatamer, shows the absence of correlation between gene copy number and level of expression.

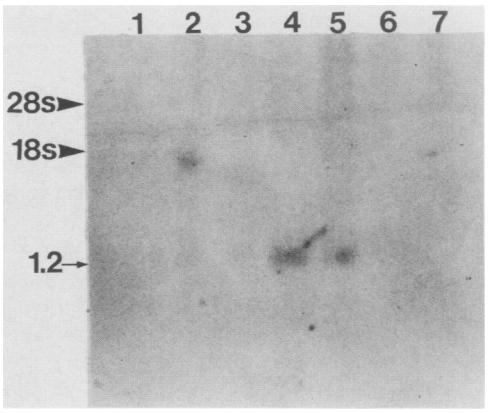


FIG. 7. Northern blot hybridization of RNA extracted from whole newborn mice and hybridized against the 810-bp *Sac*II-*Sal*I fragment of pFR400, a probe specific for foreign *dhfr* gene expression. RNA (30 μg) was loaded in each track. Lanes were loaded with the following lines: 1, P441; 2, P447; 3, 414; 4, P421; 5, P423; 6, P427; and 7, negative control. A 1.2-kilobase band corresponding to a full-length transcript of pFR400 polyadenylated at the HBV site is clearly visible in lanes 2 through 5. The positions of 28S and 18S rRNA, visualized after ethidium bromide staining, are indicated by arrows.

The results are most consistent with the interpretation that expression of the foreign gene is responsible for the defects seen. Because the integration sites are almost certainly different in each line, it is highly unlikely that insertional mutagenesis can explain the findings. It is not impossible that a family of genes, different members of which are interrupted by pFR400 insertion in each pedigree, regulates the developmental processes disturbed in our mutant animals. However, such an explanation would strongly indicate nonrandom integration of pFR400 in our animals, a hypothesis which conflicts with a large body of data indicating random integration of microinjected sequences in transgenic mice (8, 17, 26, 31). Thus the data, as well as the finding of pFR400 expression in abnormal transgenic mouse pedigrees, are best explained as based on pFR400 gene function.

The presence of SV40 and HBV DNA in the animals is unlikely to be the basis of the developmental lesions. A number of transgenic mice carrying SV40 sequences which more than encompass those introduced into our animals have now been produced. In the absence of expression of the SV40 large T antigen, which was not present on the construct used here, those animals were phenotypically normal (32). Moreover, the SV40 region present on the pFR400 clone was not transcribed. Although the HBV poly(A) sequence is transcribed (Fig. 6b and 7), the presence of a translational stop signal in the *dhfr* cDNA (Fig. 1) should preclude translation of RNA from the HBV portion of the plasmid. Furthermore, in other experiments we have produced six lines of transgenic mice containing multiple copies

of the entire HBV genome and the SV40 origin, and these animals are phenotypically normal. Thus, the best explanation for the present findings is that production of the mutant *DHFR* during ontogeny leads to the abnormalities observed at birth.

The following two factors appear to determine the tissue distribution of expression of foreign genes in transgenic mice: promoter-enhancer sequences and the chromosomal integration site (3, 4, 6, 8, 18, 20, 25–29, 31–34, 40, 42, 44–46, 48). These previous observations are also consistent with the conclusion that pFR400 expression is responsible for the abnormalities in our mice. Variable severity of similar developmental anomalies in independent lines of animals could represent SV40 promoter-directed *dhfr* gene function in the same embryonic tissues, with the level of expression modified by the integration site.

It is of interest that pFR400 is expressed efficiently in adult liver as well as in newborn mice. This fact is not inconsistent with previous studies which show moderate but clearly detectable SV40-directed gene expression in livers of transgenic mice (2, 32, 40). These other clones may not be directly comparable to pFR400, however. PFR400 carries HBV poly(A) sequences which may enhance expression in liver. In addition, the tissue distribution of pFR400 expression may be modified by the *dhfr* gene itself, as cDNAs of other types have been shown to influence the tissue distribution of expression (45).

Although the mechanism by which the abnormal DHFR might be mutagenic to mouse embryos is not known, an understanding of the abnormal kinetics of the mutant enzyme suggests an explanation. The $K_m$ of the mutant enzyme for dihydrofolic acid is 3-fold greater than that of the wild-type protein, while the dihydrofolate turnover rate is 20-fold reduced relative to that of the normal enzyme (19). Thus, the presence of a large quantity of abnormal enzyme in the cell could effectively render dihydrofolic acid unavailable for biosynthesis of thymidylate, which in turn is required for DNA synthesis. In this sense, the abnormal enzyme could act as a folate reductase inhibitor.

If the above hypothesis is correct, then interference with DNA synthesis in normal mice by any means, including inhibition of DHFR, would lead to birth defects which resemble those of our transgenic animals. In this regard, it is noteworthy that administration of thymidylate synthetase inhibitors to fetal rats induces axial skeletal deformities, growth stunting, reduced fertility, and kinked tails (30, 47). Many of these anomalies are identical to those seen in our transgenic mice. Also, administration of the DNA-alkylating agent Busulfan to fetal mice leads to tail kinks and reduced fertility (J. W. Gordon, Ph.D. thesis, Yale University, New Haven, Conn., 1978), both of which are again seen in the transgenic animals. Finally, mutations in *Drosophila melanogaster* at the rudimentary locus, which is a complex of three genes in the pyrimidine biosynthetic pathway, cause developmental anomalies characterized by abnormal wing development and female infertility (5, 11, 38). Although these data suggest that expression of the mutant *dhfr* gene caused these developmental defects, formal proof of this hypothesis has not been provided. Insertion of a cDNA cloned into an identical expression vector would be required to rule out the possibility that the addition of a normal *dhfr* gene would have similar effects.

For the pFR400 gene product to induce mutations by the same mechanism as these other agents, two requirements would have to be met. First, the pFR400 protein would have to be present in sufficient amounts to compete with the product of the two normal *dhfr* alleles for the substrate, and second, folate homeostatic mechanisms within the embryo would have to be insufficient to compensate for the alterations induced by the presence of the foreign gene product. In the tissues evaluated, the gene is clearly very active (Fig. 6 and 7). The identification of transcripts on Northern blots with a DNA probe suggest very high expression of pFR400. In previous studies of herpesvirus thymidine kinase expression in transgenic mice, very low quantities of RNA were associated with high protein production (34). RNA levels in the studies performed thus far are accordingly consistent with the possibility that large amounts of the abnormal enzyme may be present in fetal mice. At present, the mechanisms by which the embryo regulates and stabilizes the folate pathway are unknown. However, it is possible to assess the likelihood that pFR400 expression in transgenic embryos is sufficient to disrupt the pathway. Embryos from P421 transgenic females cleave slowly in vitro (Table 4), an observation consistent with thymidine synthesis inhibition. If folate reductase inhibition is actually the cause of slow cleavage in this line, it should be corrected by the administration of folinic acid (leucovorin) to the cleaving embryos. These experiments, in conjunction with DNA-RNA in situ hybridization studies of P421 embryos, are currently in progress.

These abnormal mice appear to illustrate the fact that when a biochemical pathway becomes critically important at a specific stage of development, as does the folate reductase pathway during the period of rapid embryonic cell proliferation, it may be particularly sensitive to mutations of its enzymatic loci. In such cases, even heterozygous mutations could produce phenotypic changes. Further gene transfer experiments should produce additional dominant-acting changes either by expression of donor genes or by insertional disruption of host loci. Naturally occurring dominant mutations have already been associated with foreign DNA insertion: the presence of ecotropic retroviral DNA is correlated with the semidominant lethal yellow mutation in mice (7). Studies of additional transgenic mice, as well as the molecular cloning of dominant-acting genes in humans and experimental animals, should further improve our understanding of dominant mutations.

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