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Insertion of a New Gene of Viral Origin into Bone Marrow Cells of Mice

Abstract. *DNA containing the herpes simplex virus thymidine kinase (HSVtk) gene was used to transform wild-type tk$^+$ mouse L cells to a tk^{++} status in vitro using methotrexate as a selective agent. HSVtk DNA was also used to transform mouse bone marrow cells in vitro. Transformed marrow cells injected into irradiated and methotrexate-treated recipient mice gave rise to proliferating cells which in some cases dominated the marrow population and which contained HSVtk gene sequences.*

Genetic manipulation of mammalian cells growing in tissue culture has been accomplished by various methods (*1*). Recently we described a technique for inserting genes for drug resistance into cells of living mice (*2*). In this procedure, calcium phosphate precipitates of mouse DNA containing multiple copies of the gene for dihydrofolate reductase (DHFR) were used to transform bone marrow cells to a state of increased resistance to the drug methotrexate. Transformed cells had a proliferative advantage over untransformed cells under the selective pressure of methotrexate administration. Ultimately, the transformed methotrexate-resistant cells dominated the proliferating hematopoietic cell population of the mice.

Two obvious potential uses of this gene-insertion technique are apparent for use in man: (i) induction of a higher degree of bone marrow resistance to the toxicity of anticancer drugs like methotrexate and (ii) the introduction of a new gene that, by itself, does not confer any proliferative advantage on recipient cells, by linking it to a second gene conferring drug resistance.

We set out to devise conditions in which we could use the herpes simplex virus (HSV) thymidine kinase (tk) gene as a selective marker even in tk$^+$ cells, which have the normal mammalian tk gene. We reasoned that use of appropriate levels of methotrexate and exogenous thymidine should cause cell growth to be limited by the thymidine kinase produced by the normal mammalian tk gene. Consequently, transformants carrying additional tk genes would have a selective advantage. Moreover, we noted that the HSVtk enzyme has a greater affinity for its substrate than does the normal mammalian enzyme and that this might confer an additional selective advantage.

We now describe our success in first transforming tk$^+$ mammalian tissue culture cells to drug resistance with herpesvirus tk gene in vitro and then selecting, in intact animals, bone marrow cells transformed with HSVtk gene.

We used the technique of Wigler *et al.* (*1*) to insert the herpesvirus tk gene (*3*) in tissue culture cells. Mouse L cells lacking thymidine kinase (tk$^-$) were incubated with a calcium phosphate precipitate of DNA containing the herpes tk gene in the plasmid vector pBR322 and then transferred to selective HAT medium (hypoxanthine, aminopterin, thymidine) (*1*). The anticipated results for transformation of Ltk$^-$ cells to tk$^+$ status were observed, and transformation efficiency was approximately the same as that reported by Wigler *et al.* (*1*) (Table 1). Thymidine kinase specific activity of different clones varied from 1.8 to 4.2 times the specific activity of wild-type (tk$^+$) cells.

Table 1. Thymidine kinase specific activity of mouse L cells transformed in vitro with HSVtk DNA. Clones were derived from the parent tk$^-$ L cell line or tk$^+$ wild-type L cells (NCTC) by transformation with calcium-precipitated HSVtk DNA (*1*), with 4 to 20 μg of tk DNA. The tk DNA was either in circular form in plasmid pBR322 or in plasmid that had been cut with sal and ligated (*9*). Clones of Ltk$^-$ lineage were selected in HAT medium. Cells of NCTC (wild-type) lineage were selected for in medium containing $10^{-4}M$ methotrexate and thymidine (0.5 μg/ml).

Clone	Cell lineage	Specific activity (cpm/μg)
	Untransformed	
	Ltk$^-$	0.08
	Ltk$^+$ (wild type)	4.9 to 5.5
	Transformed with HSVtk in plasmid	
207	Ltk$^-$	9.9
205	Ltk$^+$	10.3
214	Ltk$^+$	12.3
	Sal cut and ligated	
214-3	Ltk$^+$	22

In addition to transforming tk$^-$ cells to tk$^+$ status, we wished to transform wild-type (tk$^+$) cells so that they would contain additional copies of the tk gene of viral origin. Wild-type (tk$^+$) cells will grow in HAT medium by utilizing the available thymidine. Consequently, we explored a range of methotrexate and thymidine concentrations that would inhibit normal cells with a single copy of tk gene but allow cells with increased concentrations of tk to grow. These selective conditions required higher folate antagonist concentrations (methotrexate, $10^{-4}M$) and lower thymidine levels (0.5 μg/ml) than used in conventional HAT medium.

Wild-type (tk$^+$) L cells were exposed to herpesvirus tk DNA under transforming conditions and then cultured under selective conditions. A number of transformed clones were isolated, and some were grown to sufficient density to allow for measurement of thymidine kinase specific activity (Table 1) and for analysis of tk-specific gene sequences in DNA, which we identified by Southern (*4*) hybridization (data not shown). Transformation efficiency of tk$^+$→ tk^{++} varied between 0.2 and 5 colonies per 10^6 cells when 4 μg of HSVtk DNA was used. Spontaneous growth of tk$^+$ cells in the selective medium was < 1 per 10^8.

On the basis of the success of selecting for cells transformed to tk^{++} status in vitro, we undertook parallel experiments in mice. Our strategy was similar to that employed for selection of expression of the DHFR gene (*2*). Mouse bone marrow cells with a distinctive chromosomal marker (T6T6) were obtained from intact normal animals and treated in vitro with a calcium microprecipitate of herpesvirus tk gene (*3*, *5*). The treated marrow, presumed to contain a few stem cells transformed by viral gene, was mixed in a ratio of 1:1.5 with "mock" trans-

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formed syngeneic marrow cells lacking the chromosomal marker (CBA/Ca cells). These Ca cells had been incubated under transforming conditions with control DNA from salmon sperm. The cell mixture was injected into genetically compatible CBA/Ca animals that had been irradiated in order to greatly reduce endogenous hematopoiesis. The infused cells gradually restored blood cell formation in marrow-depleted recipients. During the period of reconstitution, some of the animals were treated with methotrexate. Progeny of marrow cells exposed to transforming viral DNA were identified by their distinctive chromosomal marker. If there were no selective advantage to transformed cells, then the ratio of marked to unmarked dividing hematopoietic cells would be less than 1:1.5; if, however, there were a proliferative advantage, then marked cells with increased levels of tk should be found to constitute more than 40 percent of the dividing marrow population.

In addition to this indirect means of detecting a population of hematopoietic cells transformed to methotrexate resistance, we also analyzed DNA extracted from the spleens of transformed and control mice for the presence of sequences unique to the viral tk gene.

Table 2. Karyotype analysis of marrow cells from CBA/Ca mice receiving a 1:1.5 mixture of tk-transformed T6T6 and control Ca cells. Irradiated CBA/Ca mice received portions of a 1:1.5 mixture of T6 cells transformed with herpesvirus tk in plasmid and ligated herpesvirus tk gene and Ca cells mock-transformed with salmon sperm DNA. These mice are designated primary. Recipients of marrow from primary animals are designated secondary and bear the same identifying number. Primary animal 3 was not treated with methotrexate (Mtx). Control mice received an equal mixture of mock-transformed T6 and mock-transformed Ca cells and were left untreated or were treated with methotrexate.

Recipient	Mtx treatment (days)	Karyotype (percentage of T6 cells)	Viral tk DNA sequences
Primary 1	0–32	74	Positive
Primary 2	0–47	84	
Primary 3	No Mtx*	35	
Primary 4	47–60		Positive
Secondary 4	60–89	17	Negative
Controls (20)	0–90	38 ± 10	Negative

*To day 47

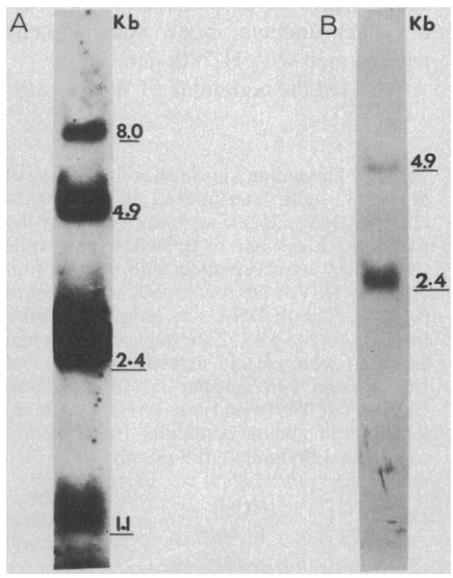

Fig. 1 Detection of HSVtk gene sequences in DNA extracted from spleen of primary mouse 4 (see Table 2). Spleen was frozen in liquid nitrogen, powdered, disrupted in isotonic buffer, and centrifuged; the nuclear pellet was digested with Pronase (Calbiochem). DNA was then purified on a $CsCl_2$ gradient. After extensive dialysis, a 20-μg portion of this DNA was digested to completion with restriction endonuclease Eco RI and subjected to electrophoresis on an 0.7 percent agarose gel. Wild-type λ bacteriophage digested with restriction endonuclease Hind III was used as a size marker. DNA was transferred to nitrocellulose paper as described by Southern (*4*) with the following modifications. (i) The DNA was nicked by exposure of the agarose gel to ultraviolet light for 4 minutes; (ii) it was transferred from agarose gel to nitrocellulose paper in a solution of 1.5*M* NaCl and 150 m*M* trisodium citrate. Probe was prepared from plasmid pBR322 containing the 3.4-kb Bam HI fragment containing the entire tk gene (*5*). It was digested with Bam HI restriction endonuclease (BRL), and the fragment containing HSVtk gene was subjected to electrophoresis on Sea Plaque agarose gel (Marine Colloids Division, FMC Corporation) and extracted with phenol. This fragment was further purified from contaminating plasmid sequences by digestion with restriction endonuclease Bgl II (BRL) and the Sea Plaque agarose gel electrophoresis and phenol extraction were repeated. The purified HSV sequences were labeled with [α-^{32}P]deoxycytidine 5′-triphosphate (*10*) to yield a specific activity of 5×10^7 cpm/μg. The nitrocellulose paper with mouse spleen DNA was treated and hybridized as described (*11*). X-ray film was exposed to the nitrocellulose paper at −70°C with the use of a DuPont Cronex Hi-Plus XJ intensifying screen. (A) Autoradiograph, 18-hour exposure. (B) Two-hour exposure of same filter strip. The film strips shown are not printed at the same magnification. No virus-specific bands were observed in DNA extracted from spleens of multiple control animals.

Simultaneous control studies were performed as follows. (i) Some mouse recipients of the mixture of transformed T6 and mock transformed Ca cells were left untreated by methotrexate. (ii) Equal numbers of untransformed T6 and Ca marrow cells were injected into Ca recipient mice, some of which were treated with methotrexate and others were left untreated. (ii) In a reversal of the usual experiment, Ca marrow cells were transformed with herpesvirus tk gene, mixed with mock transformed T6 cells, and injected into irradiated CBA/T6T6 animals.

The technique of transformation was as follows. Equal numbers of CBA/Ca and CBA/T6T6 mice were injected intraperitoneally with vinblastine (3 mg/kg) 3 days before the cells were isolated for transformation. Mitotic inhibition by this treatment is followed by a burst of stem cell proliferation (*6*). On the day of transformation, single-cell suspensions in heparinized McCoy's 5A medium with 15 percent fetal calf serum were obtained from femurs and tibias. Cells from Ca and T6 animals were placed in separate pools. Cells (5×10^7) were suspended in 10 ml of complete medium and incubated with 1 ml of calcium-precipitated DNA containing herpesvirus tk gene in plasmid form (4 μg), ligated tk gene sequences in the absence of plasmid (4 μg), and salmon sperm DNA (32 μg) as carrier. Incubation was continued for 4 hours at 37°C in 5 percent CO_2. During this period, some marrow cells adhered to the flask. A similar procedure was used for Ca cells, which were incubated with salmon sperm DNA. Loosely adherent cells were collected after incubation and centrifuged at 150*g* for 10 minutes, then resuspended in DNA-free complete medium. After careful cell counts, T6 and Ca cells were combined in a ratio of 1:1.5 and between 2×10^6 and 2×10^7 cells were injected intravenously into recipient CBA/Ca mice that had been irradiated 24 hours previously with 850 rads. The mice receiving mixtures of control Ca cells and transformed T6 cells were treated with an escalating schedule of methotrexate (*2*) for 28 to 70 days. At intervals, marrow samples were analyzed (Table 2) and injected into secondary recipient animals.

By day 32 after transformation, we observed a predominance of T6-marked cells in a dividing marrow population of a methotrexate-treated animal. Control animals receiving an equal mixture of untransformed T6 and Ca cells (1:1) had a predominance of Ca karyotype. In these control recipients, a ratio of Ca to T6 karyotype greater than 1 was anticipated

because of the contribution of endogenous CBA/Ca hematopoiesis in these recipient animals. In 20 such control animals, none showed a predominance of the T6 karyotype.

Two methotrexate-treated primary recipient animals showed a predominance of the T6 karyotype (Table 2). An untreated animal and secondary recipients of marrow from primary recipients demonstrated predominance of the Ca karyotype. In three other experiments, we observed predominance of the transformed karyotype (whether T6 or Ca was transformed) in animals followed for 30 to 60 days. Usually this predominance was not transmitted to secondary recipients, but in some cases these secondary animals had 75 to 85 percent of the transformed karyotype and had detectable viral gene sequences in hematopoietic tissues. In an occasional animals observed for 60 to 120 days, predominance of the transformed karyotype was observed even though the animals had never been treated with methotrexate. Hematopoietic tissues of these animals have not yet been examined for viral genes.

Selected primary recipient animals receiving transformed marrow were analyzed for the presence of tk enzyme and found to have viral-type pyrimidine kinase (*7*).

To demonstrate whether or not insertion of viral genes into proliferating marrow cells had occurred, we extracted DNA from spleens of selected experimental and control mice and subjected this DNA to Southern hybridization analysis (*4*).

With insertion of the complete tk gene in plasmid and subsequent complete restriction with Eco RI endonuclease, one would anticipate gene fragments consisting of 0.9, 2.4, and 4.7 kilobase pairs (kb) (*8*). Incomplete restriction would result in additional larger bands. With insertion of the ligated Bam fragment of the viral tk gene and subsequent restriction with Eco RI, one would anticipate gene fragments of 1.1, 1.2, 1.4, and 2.4 kb in size (*8*). Furthermore, the 2.4-kb band would be expected to be the most intense band, whether tk was inserted in plasmid or in ligated form. As shown in Fig. 1, A and B, tk bands of 1.1, 2.4, 4.9, and 8.0 kb were demonstrated in mouse spleen cells.

By varying the film exposure time, we demonstrated that the 1.1-kb band was wide and encompassed the fragment size 1.1 through 1.4 kb (data not shown). Control mice and secondary recipient mice from the same experiment but lacking T6 predominance failed to demonstrate herpes tk sequences. The observed bands of 1.1, 2.4, and 4.9 kb were considered to be of the expected size within the resolving power of the method. From these data we conclude that herpesvirus tk gene sequences were present in the spleen cells of this mouse. Similar sequences were identified in other mice of this and other independent experiments. The exact mode of integration of the HSVtk sequence into mouse host cell DNA remains to be determined.

Our results show that, with appropriate selective in vitro conditions, cells with multiple tk genes show a proliferative advantage over cells with a normal complement of the mammalian tk enzyme. More importantly, we have shown that analogous selective techniques can be used in intact animals. Syngeneic hematopoietic cells transformed with herpesvirus tk genes in vitro were injected into intact mice. Methotrexate administration was used to provide a proliferative advantage for cells carrying added tk genes relative to normal cells. Although we assume that only a very small percentage of the recipient cells actually acquired functional tk genes, it was possible to show that their proliferative advantage was such that they became the predominant component of the dividing marrow cell population in some mice. The spleens of these animals contained dividing hematopoietic cells. DNA extracted from these spleens was shown to contain DNA sequences of the herpesvirus tk gene.

We selected the herpesvirus tk gene for our studies because of its availability, ease of manipulation, and potential for conferring resistance to drugs that block de novo DNA synthesis. Because of its relatively small size, the tk gene has certain clear advantages over other available genes for conferring drug resistance to hematopoietic cells.

In addition, the viral tk gene can be readily linked to other cloned genes by available technology. For example, herpesvirus tk gene has been linked to the chromosomal rabbit beta globin gene presumed to contain the controller sequences involved in the initiation of transcription of the globin gene. The linked genes have been inserted into mouse tissue culture cells, which then express at least some sequences of rabbit beta globin messenger RNA (*9*). It is theoretically possible that the linked genes might also express beta globin protein when inserted into hematopoietic cells actively engaged in globin synthesis. It is clear from our studies that the techniques of inserting and selecting for expression of new genes are as applicable to intact animals as they are to tissue culture cells. It is logical, therefore, to consider their eventual use in patients with malignant diseases receiving anticancer drugs. They might also be useful in patients with severe sickle cell disease and thalassemia major. Patients with these hemoglobinopathies lack functionally normal globin genes and may be candidates for gene replacement techniques. In theory, beta or alpha globin genes could be linked to a drug-resistance gene which would then convey a proliferative advantage to bone marrow cells successfully transformed by these linked genes.

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