Gene Expression in Mice After High Efficiency Retroviral-Mediated Gene Transfer

Abstract. *A retroviral expression vector (N2) containing the selectable gene, neo^R, has been used to determine the optimal conditions for infecting murine hematopoietic progenitor cells at high efficiency. After infected bone marrow cells were introduced into lethally irradiated mice, the presence, stability, and expression of the vector DNA sequences were analyzed either in individual spleen foci 10 days later or in the blood, bone marrow, and spleens of mice 4 months later. When bone marrow cells were cultured in medium containing virus with titers of more than 10^6 colony-forming units per milliliter in the presence of purified murine interleukin-3, more than 85 percent of the resulting foci contained vector DNA. This proviral vector DNA was intact. Efficient expression of the neo^R gene was demonstrated in most of the DNA-positive foci examined. The spleens of reconstituted animals (over a long term) contained intact "vector DNA" and the blood and bone marrow expressed the neo^R gene in some animals. Thus, a retroviral vector can be used to introduce intact exogenous DNA sequences into hematopoietic stem cells with high efficiency and with substantial expression.*

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The life cycle of retroviruses makes them attractive candidates for use as agents for gene transfer. Several features are particularly relevant for their potential use for gene therapy (*1*). Recombinant retroviral "vectors" can be used to introduce new genetic material into the progenitor cells of the hematopoietic system of the mouse (*2–4*). Joyner *et al.* (*2*) detected expression of a transferred neo^R gene in individual CFU-GM colonies in vitro at an efficiency of 0.3 percent. Subsequently, Miller *et al.* (*3*) showed transfer of a functional human HPRT (hypoxanthine phosphoribosyltransferase) gene into hematopoietic stem cells that subsequently colonized the hematopoietic system of a whole mouse. Williams *et al.* (*4*), using the helper-free system that we utilize below, showed that a retroviral vector could be used to introduce DNA sequences containing a neo^R gene into about 15 percent of the CFU-S. We have characterized in vivo an efficient new retroviral vector derived from Moloney murine leukemia virus (Mo-MLV) and have determined the conditions for bone marrow gene transfer so that more than 85 percent of CFU-S are infected and the transferred gene is expressed efficiently.

The proviral form of the recombinant retrovirus N2 (Fig. 1) can produce high titer virus. Infection with this virus results in turn in a stable provirus capable of expressing genes. Vector development has been described (*5*). A large portion of the Mo-MLV coding sequence has been deleted in N2 and replaced with the bacterial neomycin-resistance gene (neo^R) which confers on eukaryotic cells resistance to the neomycin analogue G418. After transfection into the helper-free cell line ψ2 (*6*) and subsequent selection in G418, individual clones were isolated that produced N2 virus at titers ranging from less than 10^2 cfu/ml (colony-forming units) to more than 10^6 cfu/ml. More than 50 percent of isolated colonies generated virus at a titer more than 10^6 cfu/ml. When NIH-3T3 cells infected with N2 virus produced by the high-titer clone F-5B were examined by restriction enzyme analysis and Southern blotting, no evidence for deletions or rearrangements in the vector DNA was found. Furthermore, there was significant expression of neo^R-coded phosphotransferase activity in these cells.

To determine optimal time for co-cultivation of bone marrow cells with the F-5B cells, we first established the time course for viral particle production. Soon after the F-5B cells had reached confluence, the medium was changed, and the titer was measured for the next 72 hours. The effective titer increased rapidly for the first 24 hours and then continued to increase slowly over the next 48 hours. Hence, for bone marrow infections, fresh marrow cells were plated onto the F-5B cells 24 hours after a medium change.

The optimal period of time for co-cultivation of bone marrow cells with F-5B cells (Table 1) was 24 hours. Bone marrow cells were also co-cultured with F-5B cells in the presence or absence of purified growth factor interleukin-3 (IL-3) (*7*), but there was only a small beneficial effect of IL-3 (Table 1).

Since these infection efficiencies (86 percent) are greater than those reported earlier (15 percent) (*4*), the potential mouse strain specificity of these results was investigated (Table 1). A small increase in the efficiency of CFU-S infection was observed when mice of the DBA/2J strain were utilized (86 percent), in comparison to another mouse line used in retroviral studies, NFS/N mice (74 percent).

These results indicate that vector titers of more than 10^6 cfu/ml are very efficient at introducing exogenous genes into the murine hematopoietic stem cell (CFU-S) population. To determine the efficiency of bone marrow infection with lower titer virus, bone marrow cells were co-cultured with sub-confluent plates of F-5B (Table 1). In addition, bone marrow cells were co-cultivated with individual ψ2 cell clones (obtained at the same time as the higher titer clone F-5B) having lower viral titers (Table 1). No evidence of successful stem cell infection was found until the titer of the virus in the medium was 6×10^4 cfu/ml or greater (Fig. 2), regardless of whether the titer was the result of diluted, high titer cells or the total productivity of a given clone. The proportion of infected stem cells increased as the viral titer increased, with efficiencies more than 80 percent being obtained when titers were $\geq 2 \times 10^5$ cfu/ml. The effect of IL-3 was to slightly increase only both the overall proportion of CFU-S infected (Table 1 and Fig. 2) and the average number of splenic foci found after infection (9.6 versus 8.4) (Table 1).

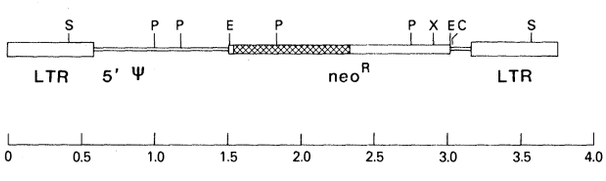
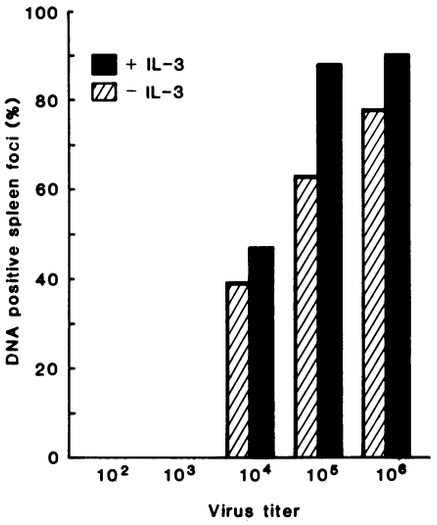


Fig. 1. Diagram of the integrated vector (proviral) N2. 0 to 1.5 and 3.0 to 3.8 kb: Moloney murine leukemia virus sequences; 1.5 to 3.0 kb box: Tn5 sequence containing the neo^R gene (Bgl I–Bam HI fragment from Tn5) (*14*); the hatched area is the coding sequence. LTR, long terminal repeat; 5′, the donor splice site at the 5′ end; ψ, packaging sequence; restriction enzyme sites: S, Sac I; P, Pst I; E, Eco RI; X, Xho I; C, Cla I.



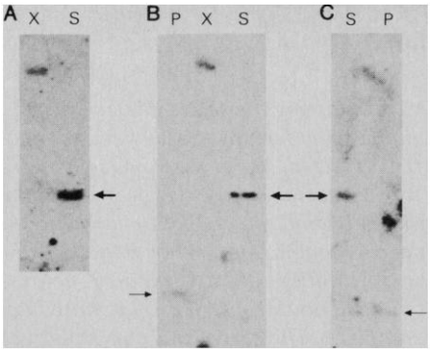


Fig. 2 (left). Titer effects upon efficiency of bone marrow infection. Indicated titers were obtained from clonal populations of various N2-producing cells as tabulated in Table 1. Culture conditions were as described in the legend to Table 1. Fig. 3 (right). Southern blots of DNA prepared from individual primary spleen foci and a whole, reconstituted spleen. Bone marrow cells were co-cultured, as described in the legend to Table 1, for 24 hours, with F-5B cells producing N2 at a titer of 2×10^6 cfu/ml. DNA from two individually isolated 10-day spleen foci obtained from infections done at different times (A and B) and from a long-term reconstituted spleen (C) was prepared as described in Table 1. The spleen DNA was prepared from a mouse 4 months after the lethally irradiated animal received 5×10^6 infected bone marrow cells. For blots A, B, and C equal amounts of DNA (30 μg per lane) were digested with restriction enzymes and then subjected to electrophoresis through 0.7 percent agarose gels. After electrophoresis, the gels were blotted (*18*) onto Biotrans filter membranes according to the suppliers' (ICN) instructions. Hybridizations were as described in the legend to Table 1, with films exposed for 5 days. Enzyme digestions were with Xho I (X), Sac I (S), and Pst I (P). Large arrows indicate the position of the 3.2-kb Sac I fragment (Fig. 1); small arrows indicate the position of the 0.9-kb Pst I fragment (Fig. 1).

Table 1. Spleen focus analyses from bone marrow infections. N2, a Moloney-based retroviral vector containing the neo^R gene, was transfected using calcium phosphate[15] into Ψ2 cells at 20 μg per 5×10^5 cells per tissue culture plate (100 mm). Permanently transfected $G418^R$ clones were isolated after 10 to 14 days selection, then individually expanded and the number of virus particles conferring G418 resistance to 3T3 cells was determined. The individual cell lines were grown to confluence at 37°C in 5 percent CO_2 in air at 100 percent humidity on tissue culture plates (100 mm) in 10 ml of NIH 3T3 medium defined as DMEM (Dubecco minimal essential medium) (Biofluids, No. 104) plus 10 percent (by volume) defined fetal bovine serum (Hyclone) plus freshly added L-glutamine (300 μg/ml). The medium was changed and then removed 24 hours later; it was centrifuged to remove floating cells, and the supernatant (containing viral particles) was frozen until the time of titration. The 3T3 cells for titration were plated (5×10^4 cells per 60-mm plate) in 4 ml of 3T3 medium, and 18 to 24 hours later the medium was removed and replaced with 1 ml of serial diluted thawed viral supernatant containing Polybrene (8 μg/ml) to increase virus adsorption. Plates were rocked every 15 minutes for 2 hours at which point 4 ml of fresh 3T3 medium was added. About 48 hours after infection the culture fluid was replaced with fresh 3T3 medium containing G418 (1 mg/ml). From 10 to 12 days later, plates were scored for $G418^R$ colonies both by microscopic counting and by methylene blue staining. Bone marrow was isolated from female DBA/2J (or NFS/N) mice (8 to 12 weeks of age). It was flushed from both femurs with α-medium (AMEM, Biofluids, No. 109). Single cell suspensions were made, cell counts were determined, and 5×10^6 cells in a volume of less than 1 ml were added to a 100-mm culture plate containing a confluent monolayer of virus-producing Ψ2 cells. The Ψ2 cells (2×10^6) had been seeded into 10 ml of 3T3 medium 48 hours earlier. Fresh 3T3 medium was added 24 hours before the addition of bone marrow cells. Individually isolated Ψ2 clones F-5B, E-1B, E-1A, E-4A, and F-2B were used to produce N2 vector at the indicated titers. Titers of 2×10^5 and 6×10^5 cfu/ml were achieved by plating the F-5B cell line at 1/10 and 1/3 the usual density. Polybrene was added to the cultures at a final concentration of 4 μg/ml. The concentration of IL-3, when added, was 20 U/ml. Purified IL-3 (*7*) had a specific activity of 0.05 ng/unit; it was diluted 1:100 into RPMI 1640 with 10 percent fetal bovine serum, filter sterilized, and kept frozen until use. Cells were cultured in 10 ml of 3T3 medium plus penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a 100 percent humidified atmosphere of 5 percent CO_2 in air. After the indicated times, bone marrow cells were recovered by gentle pipetting, and the plate was rinsed with 5 ml of α-medium. The pooled cells were sedimented and resuspended in α-medium at a final cell density of 5×10^6/ml. A cell suspension of 1×10^6 cells (0.2 ml) was injected into the tail vein of each of several syngeneic 10-week-old mice that had been irradiated with 900 rad from a ^{137}Cs source 2 to 3 hours earlier. Ten days after injection of the bone marrow cells, spleens were dissected and individual foci were isolated. Cell suspensions were made of each focus and DNA was prepared (*16*). DNA dot blot analysis of the individual foci (12 μg of DNA) was performed as described (*17*) with a nick-translated (Nick-Translation Kit, BRL) Hind III–Bgl II fragment isolated from pNeo (P-L Biochemicals). Ten positive foci were further analyzed by Southern blot analysis (*18*), and in all cases the presence of DNA of the expected restriction pattern was confirmed. The 49 foci analyzed for the +IL-3 24-hour data point were from a number of different animals infected in each of five independent experiments. The 18 foci analyzed for the +IL-3 48-hour point were from three animals infected in one experiment.

Cell line	Confluence (%)	Co-cultivation (hr)	Initial titer	Mouse strain	No IL-3						With IL-3					
					Spleens studied	Total foci	Average foci/spleen	Foci analyzed (*N*)	DNA positive (*N*)	DNA positive foci (%)	Spleens studied	Total foci	Average foci/spleen	Foci analyzed (*N*)	DNA positive (*N*)	DNA positive foci (%)
F-5B	100	24	1.2×10^6	DBA	2	10	5.0	9	7	78	8	94	11.8	49	42	86
	100	48	2×10^6	DBA	1	8	8.0	8	6	75	8	71	8.9	18	14	78
F-5B	100	24	1.0×10^6	NFS							4	38	9.5	23	17	74
F-5B	30	24	6×10^5	DBA										8	8	100
	10	24	2×10^5	DBA										16	13	81
E-1B	100	24	2×10^5	DBA	1	10	10.0	8	5	63	2	19	9.5	17	15	88
E-1A	100	24	6×10^4	DBA	6	65	10.8	38	15	39	3	32	10.7	30	14	47
E-4A	100	24	1.1×10^3	DBA	4	31	7.8	22	0	0	4	24	6.0	23	0	0
F-2B	100	24	8×10^2	DBA	3	19	6.3	12	0	0	4	40	10.0	16	0	0
			Totals		17	143	8.4	97			33	318	9.6	200		

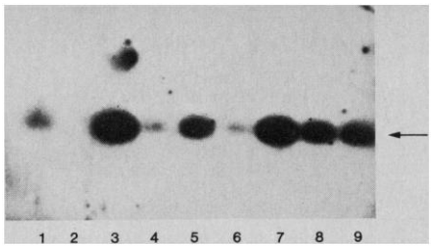


Fig. 4. Neo^R-coded phosphotransferase activity in extracts from spleen foci. Spleen foci were assayed for neomycin phosphotransferase activity (*19*). Lysates were subjected to electrophoresis on a nondenaturing polyacrylamide gel; the gel was then overlayed with agarose containing kanamycin at 25 μg/ml and 2 n*M* [γ-^{32}P]ATP (>5000 Ci/mmol). Subsequently, the gel was blotted with Whatman P81 paper. The arrow indicates the position of neo^R-coded phosphotransferase activity. (Lane 1) Lysate of 1×10^5 F-5B cells. (Lane 2) Lysate of approximately 1×10^5 uninfected spleen focus cells. (Lane 3) Lysate of 8 pooled foci from one mouse, approximately 5×10^5 cells after bone marrow infection with F-5B cells as described (legend to Table 1). (Lanes 4 to 9) Lysate of six individual foci (approximately 0.5×10^5 to 2×10^5 cells each) after bone marrow infection with F-5B cells.

In the above experiments, bone marrow cells were infected by co-cultivating them with virus-producing cells. To determine whether direct contact with virus-producing cells was required, the following infection protocols were performed: (i) infection by co-cultivation with F-5B cells; (ii) infection by co-cultivation with F-5B cells that had been washed three times with phosphate-buffered saline and then had 10 ml of fresh medium added just before addition of bone marrow cells (to reduce the starting virus titer to near zero); (iii) infection using cell-free medium derived from (ii); and (iv) infection as in (iii) but using frozen medium from F-5B cells rather than fresh. The results demonstrate that supernatant alone can infect bone marrow cells but at about one-third the efficiency obtained when F-5B cells are present (Table 2). The lower efficiency could simply be due to the lack of fresh viral particles being generated over the 24 hour co-cultivation period.

Although N2 virus was known to be stable when used to infect tissue culture cells, Southern blots were performed with DNA obtained from individual foci to test whether rearrangements or deletions of the proviral sequences occur during the proliferation and differentiation of the infected hematopoietic stem cells in vivo (Fig. 3, A and B). No evidence of gross rearrangement was detected with several restriction endonucleases. With Sac I, which digests N2 within both LTR's, releasing a fragment nearly unit length, a band of expected size (3.2 kb) (Fig. 1) was always found. When the enzyme Xho I was used, which cleaves N2 at only one internal site, several bands with different intensities were often detected on the blots when infection took place at a high viral titer. This indicated that some stem cells had been infected several times. DNA was also digested with Pst I generating the expected 0.9-kb fragment.

Southern blot analysis provides only coarse evidence for the absence of sequence alterations. However, the presence of transcripts and phosphotransferase enzyme activity would provide evidence that the transcriptional machinery of the provirus remained functional. To this end, mice were lethally irradiated and given a portion of the cells from a DNA-positive spleen focus. Spleens were removed from these secondary mice and RNA was prepared (*8*) and analyzed. A neo^R-containing fragment of the appropriate size was detected on T1 ribonuclease gels (*9*).

The neo^R gene product was assayed by its phosphotransferase activity in extracts from individual and pooled foci. The majority of foci tested demonstrated expression of the neo^R gene. All six of the individual foci (Fig. 4, lanes 4 to 9) were positive but to different extents.

Table 2. Bone marrow infection with cell-free virus-containing medium. Bone marrow cells were isolated as described in Table 1. Single cell suspensions were cultivated for 24 hours. All cultures of bone marrow contained IL-3 (20 U/ml), Polybrene (4 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were cultivated for 24 hours, recovered, and injected into lethally irradiated mice. Spleen foci were recovered after 10 days for dot blot analysis (Table 1).

Medium condition	Foci analyzed	DNA positive (No.)	DNA positive foci (%)
	With cells		
24 hour*	14	11	79
Fresh†	12	9	75
	No cells		
24 hour‡	10	3	30
Frozen 24 hour§	12	1	8

*Co-cultivation with F-5B cells producing N2 at a titer of 2×10^6 cfu/ml 24 hours after a medium change. †F-5B cells with fresh medium. The cells were washed three times with PBS and fresh medium was added just prior to the addition of bone marrow cells. ‡The bone marrow cells were plated into cell-free medium removed from the confluent F-5B cells in B. The F-5B cells had been growing in this medium for 24 hours producing a titer of 2.5×10^6. Before the bone marrow cells were added, the medium was centrifuged to remove cells and 9 ml of supernatant was placed in a separate tissue culture dish. §The bone marrow cells were plated into cell-free F-5B medium that had been stored for several weeks in liquid nitrogen after removal from confluent F-5B cells. F-5B cells had been growing in this medium for 24 hours and had a titer of 1.6×10^6 cfu/ml.

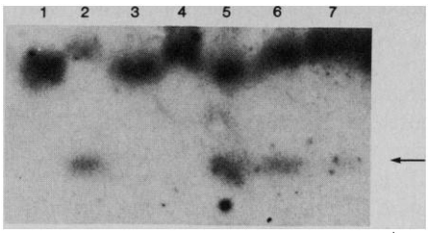


Fig. 5. Neo^R-coded phosphotransferase activity in the blood and bone marrow of four long-term reconstituted mice. (Lane 1) Lysate of 1×10^6 whole blood cells from mouse A. (Lane 2) Lysate of 1×10^6 bone marrow cells from mouse A. (Lane 3) Lysate of 1×10^6 whole blood cells from mouse B. (Lane 4) Lysate from 1×10^6 bone marrow cells from mouse B. (Lane 5) Lysate from 1×10^6 whole blood cells from mouse C. (Lane 6) Lysate from 1×10^6 bone marrow cells from mouse C. (Lane 7) 1×10^6 bone marrow cells from mouse D. The positive band in lane 7 is much clearer on longer exposures. The arrow indicates the position of neo^R-coded phosphotransferase activity. The dark slower migrating band in each lane represents a phosphotransferase activity seen to various degrees in all tissues studied, and is unrelated to the presence or absence of the neo^R gene.

The variation in activity among different foci might be partially due to varying sizes of the foci since cell numbers were not exactly equalized. More likely, however, is that the neo^R gene may be expressed at different levels in different foci, either because of multiple single-copy insertions of the N2 vector or for some other reason (such as a position effect due to the random chromosomal integration of each proviral sequence). Studies are under way to evaluate these possibilities.

To determine the long-term structural stability of N2 proviral sequences, totally reconstituted animals were obtained by injecting irradiated mice with 5×10^6 infected bone marrow cells and letting those cells repopulate the animal over a period of several months. Spleens, bone marrow, and blood were recovered and analyzed by Southern blot or phosphotransferase assays (or both). Southern blots demonstrated that the N2 sequences remained intact even after 4 months (Fig. 3C). However, the proportion of total hematopoietic cells carrying N2 may have decreased since band intensity in spleen DNA appeared to be reduced when compared with an equal amount of DNA isolated from spleen foci (compare Fig. 3, A and B, with C).

Of major interest was whether the blood and bone marrow of long-term reconstituted animals express the neo^R gene. Phosphotranferase was detected in the bone marrow of three of the four animals tested (Fig. 5, lanes 2, 6, and 7). In one animal (lane 5), a strong neo^R-

coded phosphotransferase signal was also found in the blood. Therefore, the neo^R gene is active in the circulating hematopoietic system of at least some of these animals four months after bone marrow infection and transplantation.

It is not apparent why we have obtained so much higher efficiency (even in the absence of IL-3) of infection and in vivo expression compared to the work of others. One explanation may be stability differences between N2 and the retroviral vectors used by others. A second possibility is that, although little strain difference was found here between the DBA/2J and NFS/N mouse lines, the efficiency of infection of the bone marrow of other mouse strains (for instance, C3H/HeJ[4]) is substantially different. Perhaps the 12- to 14-day foci examined by Williams *et al.* (*4*) have a lower infection frequency than the 10-day foci we studied. A final potential difference is in the reported titers of the vectors. If actual titers do differ significantly between laboratories (*3*), then marked apparent differences in bone marrow infection efficiency could be produced.

To be applicable for gene therapy, vectors such as N2 would carry additional gene sequences. Such additional sequences may have potential detrimental effects on titer. The results here indicate, however, that titers as low as 6×10^4 cfu/ml still infect murine hematopoietic stem cells with high enough efficiency to have possible therapeutic value.

In a complementary study by Keller *et al.* (*10*) it was demonstrated that the N2 vector can be found integrated and expressed in all the blood cell lineages in long-term reconstituted mice, including T and B lymphocytes. Similar studies with a slightly different vector have recently been published by Dick *et al.* (*11*).

In a number of earlier studies it was shown that retroviral vectors can undergo rearrangements and/or deletions. Using a vector derived from Friend spleen focus-forming virus, Joyner and Bernstein reported deletions of either the inserted thymidine kinase sequences or of the viral env gene (*12*). Others have reported similar problems of rearrangement in their vectors (*4, 13*). Although we have not yet sequenced the provirus, analyses such as those presented here, with Southern blots, T1 ribonuclease and neo^R gene product assays suggest an intact vector structure both during initial infection of stem cells and during subsequent in vivo stem cell proliferation and differentiation.

The utility of retroviruses as vectors for the high efficiency transfer of exogenous genetic sequences into hematopoietic cells has potential clinical relevance (*1*). A number of genetic diseases are known where the primary effect is upon the hematopoietic system. This report has established the conditions for the high efficiency transfer and expression of a gene into murine bone marrow using a new retroviral vector.

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Hematopoietic Histoincompatibility Reactions by NK Cells in Vitro: Model for Genetic Resistance to Marrow Grafts

Abstract. *In certain strains of mice, bone marrow grafts from parental donors fail to grow in first-generation hybrid mice. This "hybrid resistance" of nonsensitized F_1 hybrid mice to the engraftment of parental hematopoietic transplants contradicts the classical laws of transplantation and is dependent on a radioresistant but immunogenetically specific effector mechanism. Studies in a new in vitro model reveal that committed hematopoietic precursors of parental origin can be inactivated by direct contact with natural killer–like splenic effectors from F_1 mice. The reaction requires genetically restricted recognition, since only parental competitors syngeneic to the target bone marrow cells partially reversed this inactivation. Models of this type may be useful in studying the possible role of natural resistance in bone marrow transplantation in humans.*

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Transplants of normal and neoplastic hematopoietic cells from parental donor mice to lethally irradiated F_1 hybrid recipients are subject to an unusual host-versus-graft reaction termed hybrid resistance (*1*). This F_1 hybrid reaction to parent cells has been explained by assuming the existence of a class of noncodominant genes designated *Hh* for hematopoietic (or hybrid) histocompatibility, as opposed to the codominant histocompatibility *(H)* genes. Many of the known *Hh* genes are linked to the major histocompatibility complex in the mouse (*2*). The nature of effector cells mediating this reaction has long been a matter of speculation, as they are functional in lethally irradiated mice for at least several days, are as active in congenitally athymic mice as in euthymic littermates, and become functional only between the third and fourth weeks of life (*3, 4*).

These and other characteristics of the putative effector cells indicate that they are closely related to the effectors of natural killer (NK) activity in vitro against certain lymphoma targets (*5, 6*) [for a review on NK cells, see Herberman and Holden (*7*)]. However, this correlation between the two types of natural reactivity does not include genetic specificity, the most pertinent property of hybrid resistance (*2*). We showed earlier that the effectors of hybrid resistance in vivo are, unlike NK cells, capable of genetically restricted recognition of parental bone marrow and lymphoma cells