the CF gene location to less than 1 percent of the human genome followed by chromosomal localization will facilitate the subsequent isolation and charcterization of the gene that causes cystic fibrosis.

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Retroviral Vector-Mediated Gene Transfer into Human Hematopoietic Progenitor Cells

Abstract. *The transfer of the human gene for hypoxanthine phosphoribosyltransferase (HPRT) into human bone marrow cells was accomplished by use of a retroviral vector. The cells were infected in vitro with a replication-incompetent murine retroviral vector that carried and expressed a mutant HPRT complementary DNA. The infected cells were superinfected with a helper virus and maintained in long-term culture. The production of progeny HPRT virus by the bone marrow cells was demonstrated with a colony formation assay on cultured HPRT-deficient, ouabain-resistant murine fibroblasts. Hematopoietic progenitor cells able to form colonies of granulocytes or macrophages (or both) in semisolid medium in the presence of colony stimulating factor were present in the nonadherent cell population. Colony forming units cloned in agar and subsequently cultured in liquid medium produced progeny HPRT virus, indicating infection of this class of hematopoietic progenitor cell.*

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Defective retroviral vectors have recently been developed (*1*) for the efficient introduction of functional foreign genes into mammalian cells. Such vectors are produced by replacement of retroviral genes in cloned proviruses with foreign nonviral sequences. Hybrid vector DNA sequences, integrated and expressed in recipient cells after calcium phosphate–mediated transfection, can be rescued (pseudotyped) into transmissible viral particles by superinfection with replication-competent helper retroviruses (*1, 2*). Under optimal conditions, high titers of transmissible vector can be prepared that can then be used in vitro to infect and genetically transform virtually 100 percent of recipient cells in a population of susceptible cells. Completely helper-free transmissible vector can be produced by transfection of packaging-defective viral DNA constructs that supply helper functions in *trans* (*3*).

We have previously used retroviral vectors carrying the complementary DNA (cDNA) (*2*) for human hypoxanthine phosphoribosyltransferase (HPRT; E.C. 2.4.2.8) (*4*), to demonstrate the in vitro infection of murine bone marrow cells and the partial repopulation of the bone marrows of whole mice with these genetically transformed cells (*5*). Other groups have obtained similar results with other retroviral vectors (*6*) and have also shown that murine pluripotential hematopoietic progenitor cells can be infected by such vectors.

The ability to insert genes functionally and efficiently into human cells (*7*) allows consideration of somatic cell manipulation as therapy for certain kinds of human disease (*8*). One potential pathway toward such an application in humans would be the genetic, and therefore phenotypic, alteration of a self-renewing stem cell population in a readily accessible major organ such as the human bone

marrow. Some of the more appropriate candidates for possible treatment by such a route seem to be single gene defects with their major pathological manifestations in cells of bone marrow lineage, such as adenosine deaminase and purine nucleoside phosphorylase deficiencies. For studies of some features of retroviral gene transfer and as a model system for human gene therapy, the Lesch-Nyhan disease and its associated deficiency of HPRT activity (*4, 9*) offer major advantages. The problems and advantages of these and other model systems have been reviewed (*8*).

We have undertaken studies to determine if a foreign functional genetic marker could be introduced in normal human bone marrow stem cells (CFU_S) or the somewhat more committed granulocyte- and macrophage-producing progenitor cells (CFU_{GM}) by infecting efficiently and stably with a retroviral vector. The human HPRT retroviral gene transfer model is useful for these kinds of questions because of the existence of selection conditions available both for and against cells expressing this activity, making possible studies of long-range stability and gene shutdown that are not as feasible with other genetic markers. Not only does the HPRT retroviral vector system serve as a useful model for establishing conditions for effective gene transfer for other similarly constructed vectors, but HPRT vectors can also be used as vectors to transfer other nonselectable genes.

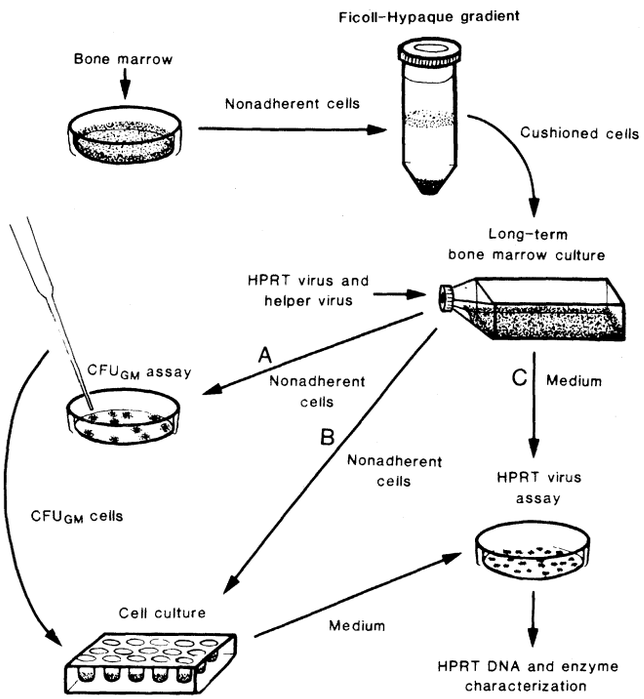

Fig. 1. Infection of cultured human bone marrow cells with HPRT virus. Heparinized human bone marrow, obtained by aspiration, was allowed to adhere to plastic for 20 minutes in order to obtain a population enriched for CFU_{GM}. Nonadherent cells were placed on a Ficoll-Hypaque gradient and the cushioned cells were cultured for up to 4 weeks in flasks. After infection with HPRT and helper viruses, the cultures were assayed for HPRT virus in (A) medium, (B) the medium from recultured nonadherent cells, and (C) the medium from cultured CFU_{GM} cells. B77 cells infected by HPRT virus formed colonies when grown in HAT and ouabain selection medium (*5*). These colonies were analyzed for the presence of human HPRT DNA and human HPRT enzyme activity.

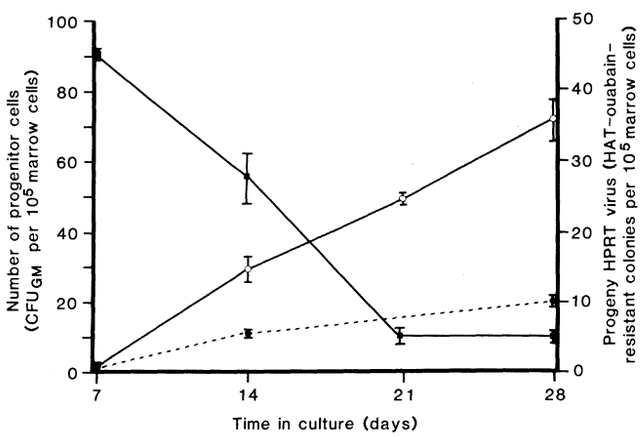

Fig. 2. Prolonged production of HPRT virus by cultured human bone marrow cells. Human bone marrow cells, prepared as in Fig. 1, were washed in McCoy's complete growth medium (*18*) plus 12.5 percent horse serum and 12.5 percent fetal bovine serum (stem cell medium). Cells (10^7) cultured in 10 ml of stem cell medium were infected with HPRT virus and a helper virus. Five milliliters of medium containing nonadherent cells was replaced by 5 ml of fresh medium every 7 days and the nonadherent cells were assayed for CFU_{GM} (■) in semisolid agar (*18*). On the tenth day colonies with more than approximately 50 cells were counted. Medium (1 ml) from the original bone marrow culture flasks (●--) or from nonadherent cells that had been recultured for 24 hours in 1.0 ml of stem cell medium and 20 percent GCT-conditioned medium (○) were assayed for HPRT virus titer by the B77 colony formation assay (*5*). Assays of recultured nonadherent cells obviate potential problems caused by interfering particles produced during the initial week in culture, and allow the detection of only freshly produced virus. It also demonstrates virus production specifically in nonadherent cells, the same population of cells from which the CFU titer is determined. Each of two determinations (bars) and their means (points) are shown. On day 7, the medium from flasks and from the recultured cells had 0.32 (0.16 to 0.48) HPRT virus particles per 10^5 marrow cells.

The scheme for the infection of the bone marrow cells and the detection of HPRT viral particles is given in Fig. 1. Human bone marrow cells were obtained from bone marrow transplantation donors or from normal volunteers by aspiration from the posterior ilium. The cultured bone marrow cells were infected with 1 ml of a helper-free, amphotropic HPRT retroviral vector (7A2). The 7A2 virus stock was derived from a clone of SCTG1 cells [an HPRT-deficient mutant of SC1 cells (*10*)] that had been cotransfected with the HPRT provirus construct pLPLM (*11*) and the plasmid pSAM, which supplies the helper virus function (*5*). The titer of 7A2 was 5×10^6 colonies per milliliter on rat 208F HPRT-deficient cells. We determined that vector 7A2 cannot replicate in the human cell line LNSV (an HPRT-deficient Lesch-Nyhan cell transformed with SV40), in mouse B77 cells or rat 208F cells by showing that infection with

3×10^6 units of virus on 2×10^6 cells does not give rise to transmissible virus in the cell supernatant.

Four hours after 7A2 infection, 0.25 ml of an N-, B-, amphotropic hybrid of Moloney-4070 component retrovirus (MA) was added as a helper virus. It was derived by transfection of plasmid pAM (*12*) into NIH 3T3 cells and had a titer of 5×10^5 colonies per milliliter on an $S^+ L^-$ assay (*13*). The infected bone marrow cultures were assayed for production of HPRT retrovirus particles by means of an HPRT-deficient, ouabain-resistant murine fibroblast line (B77) in a colony formation assay (*5*). One milliliter of medium from bone marrow cultures was used to infect 5×10^5 B77 cells. The B77 fibroblasts were grown in α minimal essential medium containing 10 percent fetal bovine serum, 2 m*M* glutamine, and gentamicin (50 μg/ml). Colonies expressing HPRT activity and therefore able to grow in the presence of $10^{-4}M$ hypoxanthine, $4 \times 10^{-7}M$ aminopterin, and $5 \times 10^{-3}M$ thymidine (HAT) plus $10^{-6}M$ ouabain were counted 11 to 14 days after infection. Transmissible HPRT virus was not detectable in culture fluid from uninfected bone marrow cells, or in infected culture fluid that did not contain bone marrow cells, when assayed on B77 fibroblasts 7 days after infection. However, when HPRT virus was used to infect cultured bone marrow cells, progeny HPRT virus could be detected beginning at 7 days after viral infection (Fig. 2). The production of HPRT virus particles by cultured bone marrow cells was detectable over a period of several weeks. There was no difference in the ultimate level of virus production when helper was added at any time between 2 hours and 6 days after infection with the defective vector.

There are several potential reasons for the apparent rise in virus production concomitant with a reduction in detectable CFU titer, the most important being that the virus production was normalized to cell number. Total virus production stayed reasonably constant, but was a product of decreasing numbers of nonadherent cells. We assume that the rising virus production per cell was caused by changing populations of producer cells resulting from differentiation of CFU_{GM}, by death of some poorly producing CFU_{GM} cells, or by gradually increasing efficiency of virus production per cell.

Since nonadherent cells could produce HPRT virus, we wished to determine if

Table 1. Production of HPRT virus by CFU_{GM}. CFU_{GM} were aspirated from semisolid agar (18), pooled, and cultured in 0.5 ml of stem cell medium plus 20 percent GCT-conditioned medium in microtiter wells for 72 hours. The media from these cells were assayed for infectious HPRT virus in the B77 colony formation assay (*5*). As each B77 colony results from the infection of a B77 cell by a single HPRT virus, the number of B77 colonies resistant to HAT and ouabain is a determinant of the number of HPRT virus particles present. Cell morphology in the CFU assay was previously correlated with colony morphology by performing Wright-Giemsa and nonspecific esterase stains on cells from aspirated CFU_{GM} (*18*) and confirmed in several colonies examined in these experiments. These numbers represent data from five experiments with two different donors.

Cell type predicted from colony morphology	Number of pooled CFU_{GM} per well	Number of HPRT virus particles per well
Mixture of colony morphologies	2	34
	5	21
	6	47
	7	56
Granulocytes and macrophages	8	15
	4	2
Macrophages	5	3
	2	0
Granulocytes	8	>100
	2	2

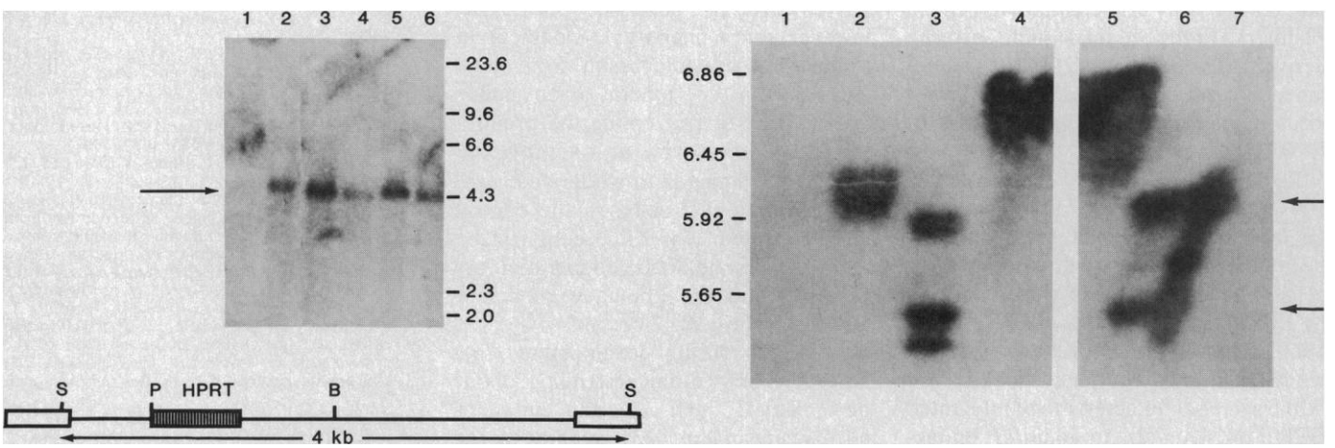

Fig. 3 (left). Analysis of DNA from B77 cells transformed to an HPRT-containing phenotype after exposure to culture media from infected human CFU_{GM} cells. HPRT-positive (HAT-resistant) colonies were expanded in culture. (Bottom) Map of the expected HPRT proviral structure, showing the long terminal repeats (open boxes), HPRT segment (box with vertical lines), and restriction sites for Sst I (S), Bam HI (B), and Pst I (P). (Top) Autoradiograph of a Southern blot (*19*) of Sst I–digested DNA hybridized to the human ^{32}P-labeled (*20*) HPRT probe. Size markers (in kb) were derived from Hind III-digested λ DNA. (Lane 1) B77 uninfected cells, (lane 2) B77 cells directly infected with 7A2 virus, (lanes 3 to 6) B77 HAT-resistant clones derived from infection with CFU_{GM} microtiter culture supernatants. Hybridization was at 68°C in 6× standard saline citrate (SSC) and washing was done in 0.1× SSC, 68°C. At this stringency, the endogenous defective murine HPRT gene sequences give no detectable signal. The arrow on the left indicates the position of the expected 4-kb HPRT-hybridizing band. Fig. 4 (right). Isoelectric focusing assay of HPRT activity in B77 cells made HAT-resistant by infection with CFU_{GM} supernatants. Cellular extracts were made, the focusing gel was run, and HPRT activity analyzed as previously described (*5, 21*). Ampholines (*p*H 5 to 8; obtained from LKB) and *p*I (E-M Science, Gibbstown, New Jersey) markers are shown on the left. (Lane 1) B77 cells, no HPRT activity; (lane 2) HeLa cells, normal human HPRT; (lane 3) B77 cells directly infected with 7A2 virus which was derived from pLPLM (*13*), altered human HPRT; (lane 4) NIH 3T3 cells, normal murine HPRT; (lane 5) murine spleen cells, normal murine HPRT; (lanes 6 and 7) B77 cells infected with CFU_{GM} supernatants. The arrows indicate the two major altered HPRT bands in B77 cells, infected either directly with HPRT virus or with CFU_{GM} supernatants.

part of the viral production was derived from progenitor cells for CFU_{GM}. To test virus infection and production by CFU_{GM}, individual colonies were grown in a semisolid agar assay with 10 percent giant cell tumor (GCT)–conditioned medium (Gibco, Grand Island, New York) as the source of colony-stimulating activity. CFU_{GM} were transferred to microtiter plates containing stem cell medium with 20 percent GCT-conditioned medium. Three different varieties of CFU_{GM} (colonies containing granulocytes or macrophages or both cell types) were tentatively identified by their morphology in agar and cultured in separate microtiter wells. All three varieties produced HPRT virus. Table 1 indicates that virus production was variable but that at least 9 out of 49 CFU_{GM} tested produced progeny virus. Comparable results were obtained in other similar experiments. We cannot say with certainty that these cells were infected by the input vector or by progeny virus produced by rescue with the helper virus, or for that matter, if the CFU_{GM} were primarily infected or were derived from previously infected and differentiating precursor stem cells. However, we can say that a high percentage of CFU_{GM} were infected and producing HPRT virus. Since the microtiter wells contained an average of approximately 75 cells from each CFU_{GM}, we calculate that the highest HPRT virus production by cultured CFU_{GM} cells was approximately one HPRT virus particle per five cells in 72 hours. Producer lines such as those derived from B77 cells (*2*), which simultaneously produced HPRT and helper viruses, had a similar maximal rate of HPRT virus production.

Recipient B77 cells were cloned as HAT- and ouabain-resistant colonies after infection with HPRT virus that had been produced by cultured CFU_{GM} cells. The infected cells were analyzed by hybridization of Sst I–digested genomic DNA to an HPRT probe (Fig. 3). All clones examined contained a 4-kilobase (kb) fragment characteristic of integrated HPRT provirus (*2*). In addition, on isoelectric focusing gels, the HPRT enzyme activity patterns from the two different human marrow–derived samples were similar but not identical to each other and to the pattern from cells directly infected with the HPRT vector (Fig. 4, lanes 6, 7, and 3). The reasons for these differences are not clear, but may be related to the formation of heteromultimers containing endogenous nonfunctional murine enzyme as well as the introduced human enzyme, as B77 cells do produce antigenically cross-reactive HPRT protein. HPRT enzyme activity patterns derived from the pLPLM gene were different from cells containing normal human and murine HPRT protein (Fig. 4). The activity in the cells infected with marrow–derived virus is different from normal human activity because of the presence of an additional, highly negatively charged COOH-terminal sequence.

These studies demonstrate that normal human bone marrow cells, including progenitor cells of the granulocyte-macrophage lineages, can be infected with a prototypical murine retroviral vector. This is in agreement with recent results (*14*) showing that wild-type murine sarcoma viruses pseudotyped with an amphotropic coat can infect human marrow cells in culture. However, the present studies demonstrate that regulatory and integration signals present in vectors derived from the nonacutely transforming murine leukemia viruses, devoid of growth factor sequences such as the *v-src* oncogene (*14*) are also suitable as vectors for the transfer of genetic information into human cells.

At a clinical level, a gene transfer model for HPRT is complicated by uncertainty (*15*) regarding the mechanism of the major neurological manifestations of the disease, including choreoathetosis, spasticity, retardation, and compulsive self-mutilation, which are not obviously related to defects in bone marrow–derived cells. The possibility does exist, however, that a phenotypic modification of the disease could result from gene expression in a peripheral organ, either by removing or preventing the production of a neurotoxin, or by providing enzyme-containing marrow-derived cells, such as microglial cells, to the central nervous system (*16*). Glial cells can be donor cells in metabolic cooperation assays (*17*) and are known to populate portions of the central nervous system at some stages during development (*16*). However, limited understanding about the biology of such cells and the exact mechanism and in vivo significance of metabolic cooperation prevents an accurate prediction of clinical benefit for intrinsic neurologic diseases.

The HPRT retroviral vector has been helpful for the development of a general retroviral gene transfer model for the study of HPRT deficiency as well as several other suitable disorders of gene expression in bone marrow cells. Successful and effective gene replacement therapy may not necessarily require introduction of a foreign gene into its usual organ or tissue site of expression. The efficiently regulated expression of a gene such as HPRT in an ectopic or unusual site may, in principle, provide a metabolically active and useful function to produce a desired phenotypic change. As a model system, HPRT provides powerful biochemical advantages for studying the stability of the gene transfer events, mobility of the integrated sequences, epigenetic mechanisms involved in the regulation of gene expression, and the expected fate and expression of other genes utilizing similar vectors.

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Isolation of a Mammalian Sequence Capable of Conferring Cell Cycle Regulation to a Heterologous Gene

Abstract. *A hybrid gene containing the 5′ sequence of a hamster histone H3 gene and the coding sequence of the bacterial neomycin-resistance gene* (neo) *was constructed. Upon transfection into the hamster fibroblast cell line K12, the hybrid gene exhibited cell cycle–dependent regulation, as evidenced by the maximal accumulation of the* neo *transcripts during synthesis of DNA in the cell cycle. In addition, cells arrested in the prereplicative phase, as a consequence of the K12 temperature-sensitive mutation, produced significantly less* neo *messenger RNA.*

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A fundamental problem in the study of cellular proliferation is the control mechanisms by which cells regulate temporal events during the cell cycle. The problem is compounded in mammalian cells because of the complexity of the genome and the general lack of well-defined cell-cycle mutants. Our approach has been to identify the elements of the mammalian genome controlling the cell cycle expression of specific sets of genes through the use of the well-studied histone gene system (*1*). Mammalian cells contain multiple copies of each of the histone genes and their variants, some of which are regulated temporally during the cell cycle at both transcriptional and post-transcriptional levels (*2, 3*). For example, the histone H3.2 gene is transcribed during the early S phase when DNA synthesis is initiated, and histone H3.2 messenger RNA (mRNA) accumulates measurably during the peak of DNA synthesis (*3, 4*). As the cells progress into late S and postreplicative (G2) phases, H3 mRNA declines correspondingly. This strict interdependence of the increased rate of histone mRNA transcription and the onset of DNA synthesis suggests that sequences in or around this cell cycle–regulated histone gene may represent specific control elements necessary for the temporal regulation of its transcription.

To test this hypothesis, we constructed a hybrid gene in which the 5′ sequence of a hamster H3 gene was fused to the bacterial neomycin-resistance gene (*neo*) (Fig. 1). We have described the subcloning of a 3.7-kilobase (kb) hamster genomic sequence containing a hamster H3 gene into the plasmid pUC8 (*3*). Partial DNA sequencing of this recombinant, pAAD3.7, revealed that the hamster H3 gene was similar to the mouse H3.2 gene (*5*). Further restriction mapping of pAAD3.7 showed that a 1.1-kb Pvu II fragment contained about 1 kb of 5′ flanking sequence plus the nucleotides encoding the first 20 amino acid residues of histone H3. This fragment was therefore isolated and tested for the presence of cell cycle–regulatory sequences. For a marker gene, we used the bacterial neomycin resistance gene (*neo*) (*6*). The use of this marker gene allowed us to discriminate expression of the exogenous chimeric gene from that of the endogenous histone H3 genes and to select stable transformants that had integrated the marker gene into their genomes at various chromosomal sites. We removed the herpes simplex virus thymidine kinase (HSV-tk) promoter from plasmid pNEO3 (*7*) and in its place inserted the hamster 1.1-kb Pvu II fragment containing the 5′ sequence of the hamster H3 gene. This hybrid gene, unlike other *neo* transfection vectors commonly used (*8*), did not contain any SV40 enhancer or promoter sequences. The only eukaryotic sequences in the hybrid gene were the hamster DNA and a short stretch of HSV-tk DNA containing the polyadenylate [poly(A)] addition site in the original pNEO3 construct. One hybrid gene, pHN/7, contained the hamster and the *neo* sequences fused in the same transcriptional orientation. This plasmid was used in all subsequent

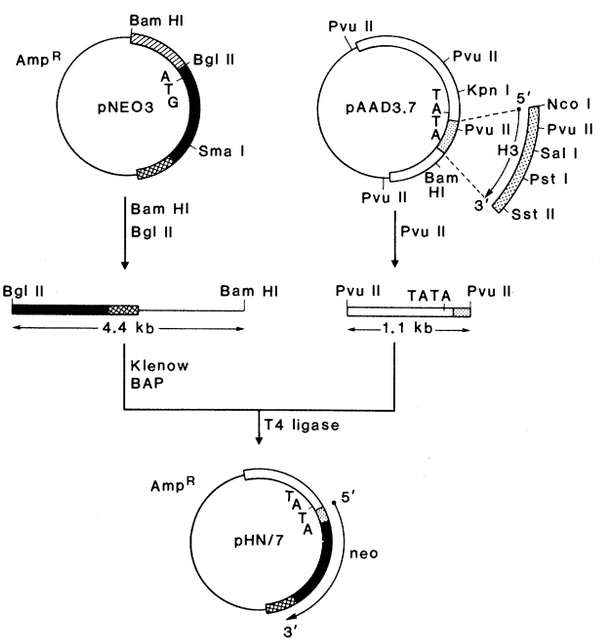

Fig. 1. Construction of the hamster histone H3-*neo* hybrid gene. The preparation of the 4.4-kb Bam HI–Bgl II fragment from the vector plasmid pNEO3 has been described (*7*). After extraction from low-melting agarose, the fragment was treated with the Klenow fragment of DNA polymerase to fill in the 5′ protruding ends generated by Bam HI and Bgl II. The fragment was subsequently treated with the bacterial alkaline phosphatase to prevent vector self-polymerization. Digestion of pAAD3.7 with Pvu II yielded four fragments with sizes of 2.4 (pUC8 vector), 1.5, 1.4, and 1.1 kb. The 1.1-kb fragment contained the 5′ flanking sequence of the histone H3 gene as well as sequences encoding for the first 20 amino acids of H3. This fragment was extracted from low-melting agarose and ligated to the *neo* vector with T4 DNA ligase. The ligated mixture was transfected into HB101, and ampicillin-resistant colonies were selected. Plasmids were prepared from individual colonies and the orientation of the hamster 1.1-kb insert with respect to the *neo* gene was determined by restriction mapping. In the plasmid, designated pHN/7, the orientation of transcription of the hamster histone fragment was the same as that of the *neo* gene, which is 1.2 kb long. If the RNA initiates at the histone promoter (TATA sequence, as indicated) and terminates at the poly(A) addition site downstream from the *neo* gene, the size of the RNA transcribed from the hybrid gene is expected to be around 1.5 kb.