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15. From distal locations in the cosmids M2A and M20A, wp's *T* and *Y* were derived (Fig. 1). With wp *T*, M9A and U10A were cloned. Restriction mapping confirmed that these cosmids overlapped M2A. Similarly, M32A, which hybridized to wp *Y*, shared diagnostic restriction fragments with M20A. The chromosome walk was continued by extension of the TNF cosmid cluster; the cosmid library was successively screened four times. From M9A, wp *U* was prepared to clone R1A and R9A. The subsequent series of cosmids, R4A, R15A, and R5A was obtained with wp *V* from R9A. From R5A, wp *W* was derived to isolate R3A, R4B, R15B, and R17A. Finally, R13C and R5B were identified with wp *X* from R17A. They were matched by another cosmid, K20A, which had previously been found with wp *V* from R9A. Therefore, a DNA segment including wp *V* must be duplicated within the cloned region. Inspection of restriction maps revealed a definitive overlap among R5B, K20A, and M32A. Thus, the genes for the TNFs and HLA-B were linked. Cosmid clones downstream from TNFα were also sought. R2A and R13B were isolated with wp *S* from O30A. A 600-bp Xba I fragment from BAT2-5 cDNA was the probe for cloning K3A, K19A, K11A, and K4B. From K4B, wp *R* was prepared to isolate K23C and K22A. Screening with wp *Q* from K22A yielded K17B and K15A, and screening with wp *P* from K15A yielded K11B, K13B, and K16A. In the last walking step, K17C, K18B, and K11C were identified with wp *O* from K16A.
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Polymerase Chain Reaction with Single-Sided Specificity: Analysis of T Cell Receptor δ Chain

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In the polymerase chain reaction (PCR), two specific oligonucleotide primers are used to amplify the sequences between them. However, this technique is not suitable for amplifying genes that encode molecules where the 5′ portion of the sequences of interest is not known, such as the T cell receptor (TCR) or immunoglobulins. Because of this limitation, a novel technique, anchored polymerase chain reaction (A-PCR), was devised that requires sequence specificity only on the 3′ end of the target fragment. It was used to analyze TCR δ chain mRNA's from human peripheral blood γδ T cells. Most of these cells had a Vδ gene segment not previously described ($V_\delta 3$), and the δ chain junctional sequences formed a discrete subpopulation compared with those previously reported.

THE POLYMERASE CHAIN REACTION (PCR) is useful in the analysis of DNA or RNA sequences from minute quantities of starting material (*1*). PCR, however, requires sequence information on both sides of the region of interest, and thus is of limited use in the analysis of sequences that have variable termini, such as those of the T cell antigen receptor (TCR) or immunoglobulins. We now show that homopolymer tailing of the variable (V) region end of T cell receptor cDNA's and the attachment of an "anchor" sequence can effectively substitute for the unknown sequence information. With an anchor primer and primers complementary to the constant (C) or joining (J) regions, T cell receptor mRNA's from small amounts of total RNA can be rapidly amplified and analyzed. We term this procedure anchored polymerase chain reaction (A-PCR) and use it to analyze the diversity of TCR δ chain mRNA's derived from human peripheral blood lymphocytes.

Most T cells in higher organisms express the αβ TCR heterodimer and are of the helper or cytotoxic subsets (*2*). Another TCR heterodimer, γδ (*3*), whose genes have been isolated and characterized (*4–6*), is present on up to 10% (*3*) of normal human peripheral blood lymphocytes of unknown function. The γδ TCR has a limited number of germline V regions available, despite a large amount of junctional diversity (*2, 6, 7*). There are 11 human V_γ genes identified, versus 50 to 100 for V_β and V_α (*8*). Only two V_δ genes have been identified in humans. $V_\delta 1$ is the predominant gene used in thymocytes, but the monoclonal antibody (MAb) TCSδ1 for $V_\delta 1$ stains only a small fraction of peripheral blood γδ T cell lines (*9*). Thus, peripheral blood γδ bearing T cells present an interesting target population for A-PCR analysis.

In this A-PCR procedure (Fig. 1), mRNA is first transcribed with reverse transcriptase and a poly(dG) tail is added to the 3′ end of the strand with terminal deoxynucleotidyl transferase (TdT). The product is then amplified with a specific 3′ primer (in this case a $J_\delta 1$ oligonucleotide) and another oligonucleotide consisting of a poly(dC) tail attached to a sequence with convenient restriction sites, termed the "anchor" (*10*). In pilot experiments, A-PCR generated a single band with known cloned cDNA sequences that had either 12 or 22 G's at the 5′ end (due to the way they had been cloned). The first round of DNA synthesis was primed with the anchor-poly(dC) primer (ANpolyC), and subsequent amplification of the poly(dG)-bearing strand was performed with either ANpolyC or the anchor (AN) primer [without the poly(dC) tail] along with the specific 3′ primer. These gave roughly equivalent results. The procedure was then tested with total RNA (3 μg) from the Molt 13 and Peer cell lines, which express δ chain mRNA. First strand synthesis was primed with either a C_δ region primer or with poly(dT). The subsequent amplification was done with ANpolyC and a J region primer (Fig. 1). When the colonies containing the amplified cDNA's were screened with a $V_\delta 1$ region probe, approximately 10% were positive. DNA from several of these colonies were sequenced, and the sequences agreed with the published sequences (*11*). This demonstrated that A-PCR can isolate the receptors from cell lines, starting with relatively small amounts of total RNA (equivalent to 10^6 cells).

We then used total RNA (~5 μg) from a polyclonal cell line, established from interleukin-2 (IL-2)–stimulated peripheral blood

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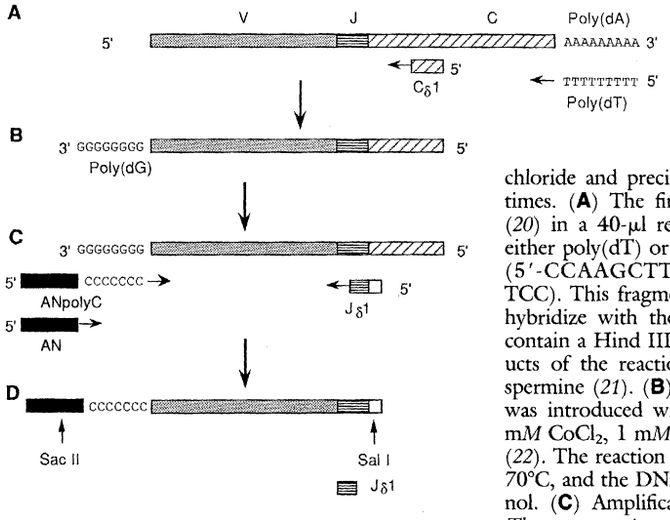

Fig. 1. Diagram of the anchored PCR technique. Total RNA (3 to 5 μg) prepared by the guinidium isothiocyanate method (*19*) was pelleted through cesium chloride and precipitated with ethanol three times. (**A**) The first strand was synthesized (*20*) in a 40-μl reaction mixture containing either poly(dT) or a specific C region primer (5′-CCAAGCTTGACAGCATTGTACTTCC). This fragment was designed to cross-hybridize with the mouse sequence and to contain a Hind III restriction site. The products of the reaction were precipitated with spermine (*21*). (**B**) A poly(dG) tail sequence was introduced with TdT (Pharmacia) in 2 m*M* $CoCl_2$, 1 m*M* dGTP for 1 hour at 37°C (*22*). The reaction was stopped by heating to 70°C, and the DNA was precipitated in ethanol. (**C**) Amplification was performed with *Thermus aquaticus* polymerase (Perkin-Elmer Cetus) in 100 μl of the standard buffer. The primers included one specific for $J_\delta 1$ (5′-GGGTCGACTTACTTGGTTCCACAGTCAC). The $J_\delta 1$ primer was designed to cross-hybridize with the mouse sequence and to contain a Sal I site. Because the region of homology to the cDNA sequence was only 16 bp, the first five cycles of amplification were annealed at 45°C with subsequent cycles annealed at 55°C. The denaturation step was at 94°C for 1 min, the annealing step was for 1.5 min, and the extension was at 72°C for 2.5 min. The primers for the poly(dG) end were a mixture of the ANpolyC primer (5′-GCATGCGCGCGGCCGCGGAGGCCCCCCCCCCCCCC) and the AN primer (5′-GCATGCGCGCCGGCCGCGGAGGCC) at a ratio of 1:9. These primers included the restriction sites Sac II, Sph I, Not I, and Sfi I. Amplification was performed for 25 cycles and the product was precipitated with ethanol. One-tenth of the sample was separated on a 1% low-melting agarose gel (Bethesda Research Labs) in tris-acetate buffer. An appropriate size fraction was cut from the gel and approximately 5% of the agarose-containing band was directly reamplified with the $J_\delta 1$ primer and AN. The reaction mixture was then cooled and the supernatant, which was separated from the agarose by centrifugation, was precipitated with spermine. (**D**) The DNA was sequentially cut with Sac II and Sal I. The DNA was separated on a 1% low-melting agarose gel, and the band from approximately 200 to 500 bp was ligated into Bluescript SK^+ (Stratagene) and transformed into bacteria (DH5α). The white-blue color screen for inserts was not reliable because many blue colonies contained the appropriate inserts. The colonies were screened with a second $J_\delta 1$ oligonucleotide (5′-CCGACAAACTCGTCTTTGGA), and positive colonies were sequenced with the miniprep technique (*23*).

lymphocytes, that expresses γδ TCR's. At most, 10% of this cell line expressed $V_\delta 1$ by immunofluorescence analysis with a MAb recognizing $V_\delta 1$ (TCS δ1), and the remaining cells expressed δ chains with unknown V regions (*9*). After the A-PCR procedure was performed, the colonies were screened with a 5′ $J_\delta 1$ oligonucleotide, and approximately one-third of the colonies were positive. From approximately one-tenth of the amplified material, over 200 positive colonies were obtained. Twenty VDJ rearrangements were sequenced, and each was unique, indicating the polyclonality of the population of cells studied (Fig. 2A) and the lack of bias toward any one sequence in the A-PCR procedure. [Several cloning artifacts were observed, however (*12*).] All of the sequences were in-frame, in contrast to the δ sequences of αβ T cells from the thymus in which two-thirds are out-of-frame (*6*). This suggests that most δ transcripts from γδ cells come from the coding allele or from a nonrearranged allele (*12*); if the noncoding allele is rearranged, it is not transcribed to any significant extent. In addition, this set of sequences rarely contained either $D_\delta 1$ or $D_\delta 2$, again in contrast to the δ sequences of αβ cells from the thymus, which frequently use multiple D regions (*6*).

Only two different V regions were seen in the 20 sequences examined (Fig. 2A). Two sequences contained $V_\delta 1$, and 18 had a previously undescribed V region, termed $V_\delta 3$ (Fig. 2B). $V_\delta 3$ does not closely resemble V_α (*13*), $V_\delta 1$, or $V_\delta 2$, whose germline gene is 3′ to the C_δ region (*6*). The most closely related mouse V region is $V_\delta 1$ (VM9), but there are only 33 of 94 amino acid identities (compared to a 70% identity between human $V_\delta 2$ and mouse $V_\delta 6$). An extra cysteine residue two residues away from the cysteine usually used in intrachain disulfide linkage is similar to the case of a mouse V_β gene segment [$V_\delta 2$ (*14*)]. Perhaps either cysteine can be used for the intrachain bridge, thus leaving a free sulfhydryl group.

Thus, a γδ cell line established from peripheral blood expressed $V_\delta 3$ in most δ chains that contained $J_\delta 1$, which is the predominant J region in both peripheral blood and thymic γδ cells (*15, 16*). Several points argue that these results can be generalized to fresh (uncultured) γδ cells and to other individuals. (i) The relative proportion of cells expressing $V_\delta 1$ is preserved between fresh and IL-2 cultured cells from thymocytes (*9*). (ii) With a $V_\delta 3$ probe, we examined several γδ cell lines from different individuals with Northern gel analysis (Fig. 3A). The presence of strong hybridization to all peripheral blood but not thymic γδ cell lines suggests that $V_\delta 3$ is expressed primarily in the peripheral blood. In contrast, when the RNA blot was rehybridized with a $V_\delta 1$ probe both thymic and peripheral blood γδ TCR's contained $V_\delta 1$, as predicted by the sequence data. (iii) Probing a Southern blot of another peripheral blood γδ cell line with both a $V_\delta 3$ and a $J_\delta 1$ probe revealed that the darkest rearranged $J_\delta 1$ band hybridized to $V_\delta 3$ (Fig. 3B), again suggesting that $V_\delta 3$ was predominant in those cells as well. The presence of another strong rearranged band, as well as other weak bands, suggests that still more V regions may be expressed. A faint rearranged band that was positive with $V_\delta 3$ but not $J_\delta 1$ raises the possibility that $V_\delta 3$ recombined with another J region. Thus we conclude that the V region diversity of peripheral blood γδ cells is limited and differs from that of the thymus. This indicates that the specificity of γδ cells in the periphery may be different from most of those found in the thymus.

With respect to possible errors produced by the amplification procedure, we have observed only one substitution (C to G) in approximately 1500 bp surveyed, and no other type of error was seen. When it is critical that every base pair be correct, verification could be achieved from two independent amplifications or from resequencing the unknown sample directly with conventional PCR after V region usage has been determined by A-PCR. A potential source of error in surveying populations of sequences as we have done is selection for or against a particular sequence. This may occur to some extent (*17*), but thus far no bias has been apparent. In particular, the amount of $V_\delta 1$ bearing sequences obtained here (10%) was identical to the proportion of $V_\delta 1$ bearing cells in the original cell line. Another potential problem is that of mixed sequences, where an incompletely replicated copy can prime the replication of a homologous member of the family, thus creating a hybrid molecule. We have not yet observed this event, and one could take steps to avoid this possibility by maintaining a large excess of oligonucleotide primer over the amplified product and by allowing a sufficient time for the extension step. Also of interest is the sensitivity of this technique. In the experiments described, we used several micrograms of RNA, equivalent to approximately

A

	< V	| N?	$D_\delta 1$	N?	$D_\delta 2$	N?	$D_\delta 3$	N	| J >	
Germline D_δ			GAAATAGT		CCTTCCTAC		ACTGGGGGATACG			
PT6	(V_δ1) ...	TGTGCTCTTGGGGAA	TGGACG				CTGGGGGATA	TTGTAGT	CACCGATAAA	+
PT10	(V_δ1) ...	TGTGCTCTTGGGCAAC	CTG		CCTT			GGTGCGTGTGAC	CACCGATAAA	+
PT1	(V_δ3) ...	TGTGCCTGTGACACC	TT				ACTGGGGGA	CCCGT	ACACCGATAAA	+
PT2	(V_δ3) ...	TGTGCCTGTGAC	CCCGT				ACTG	CCCGTCC	CCGATAAA	+
PT3	(V_δ3) ...	TGTGCCTGTGACA	A		CCT	G	CTGGGGGATAC	ATATG	ACACCGATAAA	+
PT4	(V_δ3) ...	TGTGCCTGTGACA	TGG		TTCC	CTCACCCATTCGA	ACTGGGG	TCATCTTTCCGGG	TAAA	+
PT5	(V_δ3) ...	TGTGCCTGTGAC	CT				ACTGGGGGATACG		ACCGATAAA	+
PT7	(V_δ3) ...	TGTGCCTGTGACACC	TTAT				CTGGGGGATACG	AGGGTCGG	ACCGATAAA	+
PT8	(V_δ3) ...	TGTGCCTGTGA	TATTATT				ACTGGGGGA	CAAAGGATGT	ACACCGATAAA	+
PT9	(V_δ3) ...	TGTGCCTGTGACACC	GTC	GAA			ACTGGGGGATAC	T	ACACCGATAAA	+
PT11	(V_δ3) ...	TGTGCCTGTGACACC	GGAGGGTT				ACTGGGGG	GCGAA	ACCGATAAA	+
PT12	(V_δ3) ...	TGTGCCTGTGAC	GGGGTGGGG				ACTGGGGGATACG	CGG	CCGATAAA	+
PT13	(V_δ3) ...	TGTGCCTGTGAC	CGGG				TGGGG	AGGT	ACACCGATAAA	+
PT14	(V_δ3) ...	TGTGCCTGTGAC	TCT				TGGGGG	G	ACACCGATAAA	+
PT15	(V_δ3) ...	TGTGCCTGTGAC	CCCC		TCC	GT	ACTGGGGGATAC	A	ACACCGATAAA	+
PT16	(V_δ3) ...	TGTGCCTGTGAC	CA				ACTGGGGGATAC	TCC	CACCGATAAA	+
PT17	(V_δ3) ...	TGTGCCTGTGACACC	GGAGGAT				ACTGGG		CCGATAAA	+
PT18	(V_δ3) ...	TGTGCCTGTGACACC					CTGGGGGAT	CAGGTT	ACCGATAAA	+
PT19	(V_δ3) ...	TGTGCCTGTGAC	T		CCTT		ACTGGGGGA		CACCGATAAA	+
PT20	(V_δ3) ...	TGTGCCTGTGAC	CCT		CTT	GGCTCAAGT	ACTGGGGGATAC	CCGGC	CACCGATAAA	+

Fig. 2. (**A**) Junctional region sequences of a set of cDNA sequences from a peripheral blood γδ cell line. The $V_\delta 3$ to N region joints are hypothetical since the germline $V_\delta 3$ sequence is not yet available. Homologies to the genomic D regions of at least 3 bp are underlined. The + signifies that the sequences are in frame between the V and J regions. The sequences 5′ to the junctional sequences shown depend on the V region and begin with 14 to 20 C's, while the sequences 3′ of the region shown contain the rest of the J region and part of the $J_\delta 1$ primer. The sizes of the complete cDNAs varied from approximately 100 to 450 bp. If necessary, a set of longer clones could have been established with more stringent size selection. (**B**) Deduced amino acid sequence of PT11, which includes a full length $V_\delta 3$ (*24*). The putative leader sequence is based on homology to other leader sequences. The cysteine residues are boxed. The nucleotide sequence is available upon request and has been deposited at GenBank (accession number M21784).

B

```
-19          Leader              -1 | 1   V Region   10
M Q R I S S L I H L S L F W A G V M S | A I E L V P E H Q T
                  20                  30                  40
V P V S I G V P A T L R C S M K G E A I G N Y Y I N W Y R K
                  50                  60                  70
T Q G N T M T F I Y R E K D I Y G P G F K D N F Q G D I D I
                  80   V Region       90                  100
A K N L A V L K I L A P S E R D E G S Y Y C A C D T | G G L L
                J Region
G G E | T D K L I F
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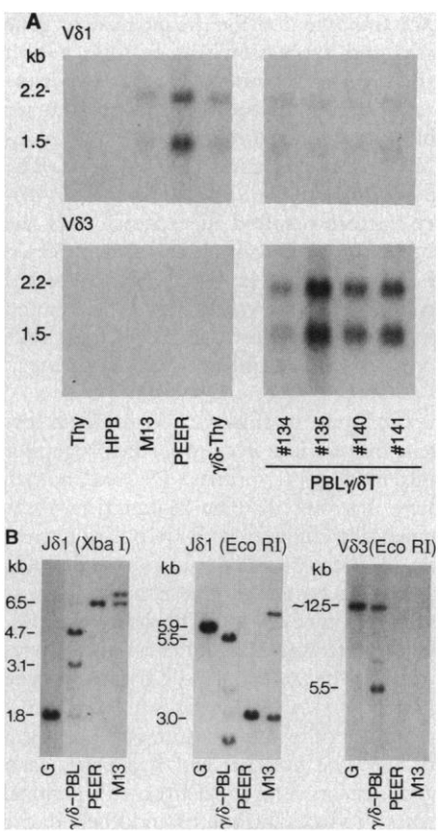

Fig. 3. (**A**) Northern analysis of peripheral blood γδ cell lines. The top panels were probed with a $V_\delta 1$ probe (*6*). The bottom panels were the same blots probed with a $V_\delta 3$ probe (PT11 cut with Sac II and Hpa II, thus deleting the J region sequences). G, granulocyte DNA; HPB, an αβ expressing leukemic cell line; M13 and PEER, γδ expressing leukemic cell lines; γδ-THY, a thymic γδ expressing cell line; 134, 135, 140, and 141, γδ expressing cell lines from the peripheral blood. (**B**) DNA analysis of a peripheral blood γδ cell line. The $J_\delta 1$ probe was as described (*6*), and the $V_\delta 3$ probe was as in Fig. 2. Both probes were hybridized to the same blots. The 4.7-kb band in the Xba I cut DNA hybridized to the $V_\delta 3$ probe (*25*), while the 5.5-kb band in the Eco RI cut DNA hybridized to $V_\delta 3$. The Xba I 6.5-kb band and the 3.0-kb Eco RI band hybridize to $V_\delta 1$ (*25*). $V_\delta 3$ appears as a single band in unrearranged DNA cut with three different restriction enzymes and it is deleted in two tumor lines (Peer and Molt 13) which use $V_\delta 1$. Thus it is likely that $V_\delta 3$ is a single copy gene segment(as are $V_\delta 1$ and $V_\delta 2$) and that it is 3′ of $V_\delta 1$.

a million cells, and obtained 2000 TCR δ containing subclones. Conventional PCR has been used successfully on RNA derived from a single cell and A-PCR potentially could approach that level of sensitivity.

Variations of the A-PCR procedure may be necessary depending on circumstances. The method of RNA preparation, the conditions and number of cycles of amplification, and the oligonucleotides used are all variables. The central benefit of A-PCR is that specificity need not be provided at both ends of the molecule to be amplified. Specificity can be provided at one end by nested oligonucleotides, used either in amplification or in selection from the amplified clones. The cloning of any TCR or antibody gene from cell clones or tumor material should be easier with A-PCR. In particular, experiments otherwise difficult to do may now be possible, such as the survey of antigen receptors from different tissues or the isolation of receptors from small numbers of cells.

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26. We thank D. Denny for suggesting the sequence of the anchor polylinker. Supported by a Hulda Irene Duggan Investigator Award from the Arthritis Foundation (E.Y.L.), a Centennial Fellowship from the MRC of Canada (J.F.E.), the PEW Foundation (M.M.D.), and grants from the National Institute of Allergy and Infectious Disease (M.M.D.).

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High-Level Recombinant Gene Expression in Rabbit Endothelial Cells Transduced by Retroviral Vectors

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By virtue of its immediate contact with the circulating blood, the endothelium provides an attractive target for retroviral vector transduction for the purpose of gene therapy. To see whether efficient gene transfer and expression was feasible, rabbit aortic endothelial cells were infected with three Moloney murine leukemia virus–derived retroviral vectors. Two of these vectors carry genes encoding products that are not secreted: N2, containing only the selectable marker gene *neo*^{R}, and SAX, containing both neo^R gene and an SV40-promoted adenosine deaminase (ADA) gene. The third vector, G2N, contains a secretory rat growth hormone (rGH) gene and an SV40-promoted neo^R gene. Infection with all three vectors resulted in expression of the respective genes. A high level of human ADA expression was observed in infected endothelial cell populations both before and after selection in G418. G2N-infected rabbit aortic endothelial cells that were grown on a synthetic vascular graft continued to secrete rGH into the culture medium. These studies suggest that endothelial cells may serve as vehicles for the introduction in vivo of functioning recombinant genes.

THE USE OF RETROVIRAL GENE transfer over the past decade has greatly facilitated the transfer of genetic material into mammalian cells. Retroviruses have a high efficiency of infection, stable integration, and expression in most cells (*1*). Nonimmortalized cells, which may be transfected at low efficiency (or not at all) by physical and chemical means, are very amenable to retroviral-mediated gene transfer. Although bone marrow hematopoietic progenitor cells have been frequently targeted for retroviral-mediated gene transfer, these cells suffer from drawbacks such as the variable ability to express certain genes in mouse bone marrow (*2, 3*) or inefficient gene transfer into hematopoietic progenitor cells in dog (*4*), primate (*5*), and human bone marrow (*6*). The requirement for a genetically engineered cell population capable of both long-term survival and stable expression has led to the consideration of other cell types such as fibroblasts (*7–10*), lymphocytes (*11, 12*), human keratinocytes (*13*), and hepatocytes (*14*) for use in gene therapy.

The endothelium, because of its contiguity with the bloodstream, is a particularly attractive target for the delivery of functional genes in vivo. The use of endothelium for gene transfer would permit secretion of a recombinant protein from genetically engineered endothelial cells directly into the blood. Alternatively, endothelial cells expressing a nonsecreted recombinant protein might be able to inactivate a toxic substance that is circulating in the blood.

To determine the efficacy of retroviral-mediated gene transfer while assessing the expression of genes encoding secreted and nonsecreted products, cultured rabbit aortic endothelial cells (RAEC) were infected with three recombinant retroviral vectors containing the neomycin resistance (neo^R) gene alone or in combination with either the adenosine deaminase (ADA) gene or the rat growth hormone (rGH) gene (Fig. 1).

Before infection, monolayers of RAEC (*15*) were characterized by morphology, uptake of diacetylated low density lipoprotein (dil-ac-LDL) (*16*), and by the presence of angiotensin-converting enzyme activity (4.1×10^5 molecules per cell) (*17*). Retroviral packaging lines were made that contained SAX, N2, or G2N (*18–21*). Vector-containing viral supernatants that exhibited titers of 5×10^5 colony-forming units per milliliter or greater were used for infection. After infection and selection in G418-containing

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