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Use of an Epstein–Barr virus episomal replicon for anti-sense RNA-mediated gene inhibition in a human cytotoxic T-cell clone

(CD8)

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ABSTRACT **A methodology was developed for stable gene transfer into cloned nontransformed human T lymphocytes. Stable high-level gene expression was achieved in cloned human T cells by using a self-replicating Epstein–Barr virus (EBV) episomal replicon. A comparison of five eukaryotic promoters established that the Rous sarcoma virus 3′ long terminal repeat (RSV 3′ LTR) and the lymphopapilloma virus (LPV) 5′ LTR are optimal for episome-based expression in T cells. Effective (>95%), selective, and reversible anti-sense RNA-mediated gene inhibition of a model T-cell-associated molecule (CD8) was achieved in a cytotoxic human T-cell clone by using an EBV episome-based, RSV 3′ LTR-driven expression system. The linking of anti-sense RNA mutagenesis and T-cell cloning technologies should contribute significantly to studies of human T-cell function.**

Gene transfection offers a powerful experimental approach for defining the functional roles of specific molecules in T lymphocytes. Stable gene transfer has been accomplished for T-cell tumor lines (1–4), T-cell hybridomas (5, 6), cord blood lymphocytes (7), and cloned murine T lymphocytes (8–10) but has not been described for cloned human T lymphocytes. Unlike T-cell tumor lines and hybridomas, T-cell clones possess normal karyotypes; are regulated in their proliferation by antigen, lymphokine secretion, and lymphokine receptors; and readily mediate specific and nonspecific cytotoxicity (11). Hence, the availability of transfection mutants of cloned, human T-lymphocyte lines would contribute significantly to molecular studies of human T-cell function.

Plus-sense transfection analysis has been applied to the study of a variety of T-cell-associated molecules such as the T-cell antigen receptor (5), the interleukin 2 (IL-2) receptor (8), Thy-1.2 (1), CD8 (6), CD4 (12), CD7 (13), and interferon-γ (9). However, the anti-sense RNA mutational approach (14) has not been used in studies of T lymphocytes. Anti-sense RNA technology, by enabling selective gene inhibition, provides an alternative transfection strategy in which deletional mutants can be used to determine whether specific molecules play obligatory roles in defined cellular functions.

Our goal in the present study was to develop an efficient system for generating anti-sense RNA mutants of cloned human T lymphocytes. To this end, we have explored the utility of a high copy number Epstein–Barr virus (EBV) episomal replicon (15) to serve as a vector for high level stable expression of sense and anti-sense RNA transcripts. Episomal replicons are circular DNA elements designed to self-propagate extrachromosomally in eukaryotic cells. As expression vectors, episomal replicons offer a means for amplifying gene copy number in cells and, furthermore, circumvent complications that arise from chromosomal integration—e.g., position effects on levels of transcription and insertional mutagenesis of host cell sequences at the integration site. The EBV origin of replication (oriP) and the EBV nuclear antigen 1 together confer an episomal replication capacity to circular DNA elements in an array of human cell types, including lymphoid cells (15). Here we describe a methodology for stable gene transfer into cloned nontransformed human T lymphocytes by using an EBV episomal replicon, and we identify promoters suitable for episome-based expression in these cells. In addition, we establish the utility of episomal vectors for anti-sense RNA-mediated gene inhibition of a model surface glycoprotein, CD8, on a human cytotoxic T-cell clone.

MATERIALS AND METHODS

Reagents. Complete medium consisted of RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), L-glutamine (2 mM), Hepes (10 mM), penicillin (5 units/ml), and streptomycin (5 μg/ml). Recombinant DNA enzymes were obtained from New England Biolabs, Boehringer Mannheim, and Pharmacia. Other reagents were obtained from the indicated sources: chloramphenicol and lysozyme (Sigma); polyethylene glycol 1540 (Koch Light); hygromycin B and A23187 (Calbiochem; A23187 stock solution: 0.7 mM in ethanol); [^{14}C]chloramphenicol (New England Nuclear); acetyl CoA lithium salt (Pharmacia).

Cells. Two independently derived nontransformed (IL-2 dependent) human T-cell clones, V1 ($CD4^+CD8^-$) and 8L2 ($CD4^-CD8^+$), were used for these studies. The derivation of the influenza hemagglutinin-specific HLA-DR5-restricted cytotoxic cell line V1 has been described (16, 17). The 8L2 line was cloned by micromanipulation of single cells (Autoclone, EPICS V, Coulter) from mixed lymphocyte cultures in 96-well microtiter plates containing 200 μl of complete medium per well. To each well we added 20,000 γ-irradiated (5000 rads; 1 rad = 0.01 Gy) allogeneic human peripheral blood mononuclear cells (PBMC) and 10% MLA-144 culture supernatant (CS) as a source of IL-2. The growth cycle of both of these clones is regulated and characterized by transient expression of IL-2 receptors (16). Clones were stimulated weekly with OKT3 (anti-CD3) monoclonal antibody (mAb; 1 ng/ml; Ortho Diagnostics), IL-2 (10% MLA-144 CS), and irradiated allogeneic human PBMC (0.75×10^6 cells per ml). After 3 days of stimulation, clones were washed

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Abbreviations: EBV, Epstein–Barr virus; RSV 3′ LTR, Rous sarcoma virus 3′ long terminal repeat; LPV, lymphopapilloma virus; IL-2, interleukin 2; oriP, EBV origin of replication; PBMC, peripheral blood mononuclear cells; CS, culture supernatant; mAb, monoclonal antibody; CAT, chloramphenicol acetyltransferase; PHA, phytohemagglutinin.

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and subcultured in fresh complete medium supplemented with IL-2 (10% MLA-144 CS) for 4 days prior to restimulation (Fig. 1).

Plasmid Constructions. To assemble promoter–chloramphenicol acetyltransferase (CAT)/220.2 plasmids (Fig. 2*a*), we obtained promoter–CAT plasmids from several investigators: SV2CAT (simian virus 40 early promoter; B. Howard; ref. 18); RSVCAT [Rous sarcoma virus 3′ long terminal repeat (RSV 3′ LTR); B. Howard; ref. 19]; LPV-5′-CAT [lymphopapilloma virus (LPV) 5′ LTR; R. Miksicek; ref. 20]; I10CAT (rat GRP78 gene calcium ionophore-inducible promoter; A. Lee; ref. 21); HS1CAT (phMT-II_A-CAT; human heavy metal-inducible metallothionein II_A promoter region encompassing ≈850 base pairs of sequence upstream of the $hMTII_A$ gene; M. Karin and A. Haslinger; ref. 22); and SV0CAT (no eukaryotic promoter; B. Howard; ref. 18). p220.2 was generously provided to us by B. Sugden (Madison, WI). This 8.9-kilobase plasmid is a derivative of p201 (15) in which a polylinker (*Bam*HI, *Xba* I, *Sal* I, *Pst* I, *Hin*dIII) has been inserted at the *Nar* I site within the herpes simplex virus 1 thymidine kinase gene termination sequence (B. Sugden, personal communication; see Fig. 2*a*). For construction of promoter–CAT/220.2 plasmids, a second *Bam*HI site was first introduced at unique restriction sites upstream of the promoter in promoter–CAT plasmids using *Bam*HI linkers (New England Biolabs), and promoter–CAT cartridges were then mobilized by *Bam*HI digestion and inserted in both orientations into the unique *Bam*HI site of p220.2 (C.A.H. and M.L.T., unpublished data).

pT8F1 (generously provided to us by R. Axel; ref. 23), a cDNA plasmid encompassing the complete coding sequence of human CD8, was the source for the CD8 DNA segment in the anti-sense CD8 construct α-CD8/REP1 (see Fig. 3 and *Results*). RSVPA1, a plasmid in which the RSV 3′ LTR (derived from pRSVCAT), *Eco*RV and *Bam*HI subcloning sites, and the simian virus 40 late polyadenylylation/termination sequence (derived from pcDV1; ref. 24) are sequentially arrayed, was assembled by us in a multistep procedure (R.K.G., H.K.S., and M.L.T., unpublished data).

Transfection Procedures. Electroporation (used here for promoter–CAT/220.2 episomes) was performed with a Promega Biotec X-Cell 450 electroporator at 200 V, 1100 μF, 0.7 sec time load, in phosphate-buffered saline (PBS) containing 20 mM Hepes (pH 7.2), 500 μg of sheared salmon sperm DNA as carrier, and 20 μg of plasmid DNA. The protocol for protoplast fusion (used here for the α-CD8/REP1 episome) has been described (25), and we have introduced modifications for suspension cells. Protoplasts were copelleted with 10^7 logarithmically growing target cells (prewashed with PBS three times) (5000:1, protoplast/target cell ratio) at 1700 × *g* in a DuPont-Sorvall HS-4 centrifuge for 20 min at 4°C. Fifty percent polyethylene glycol 1540 in PBS (1 ml) was added dropwise over 1 min, and cells were then immediately diluted with PBS (1 ml) over 1 min and more PBS (20 ml) over 3 min, pelleted, washed twice with PBS, and resuspended in six-well plates (10^6 cells per ml) in complete medium supplemented with gentamicin (200 μg/ml) and IL-2. Stable transfectant lines corresponding to individual wells were derived by the

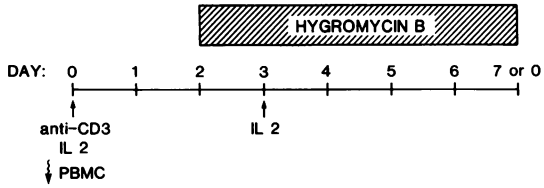

FIG. 1. Stable gene transfer into human T-cell clones. A 7-day cycling scheme was used to derive and maintain cloned human T-cell transfectants. Cells were washed and stimulated at the start of the cycle (day 0) with anti-CD3 (OKT3) mAb, IL-2 (10% MLA CS), and irradiated PBMC, and washed and restimulated (with exogenous IL-2 only) on day 3. Hygromycin B (0.15 mg/ml) was added to the culture medium on day 2 and again on day 3 (after the wash).

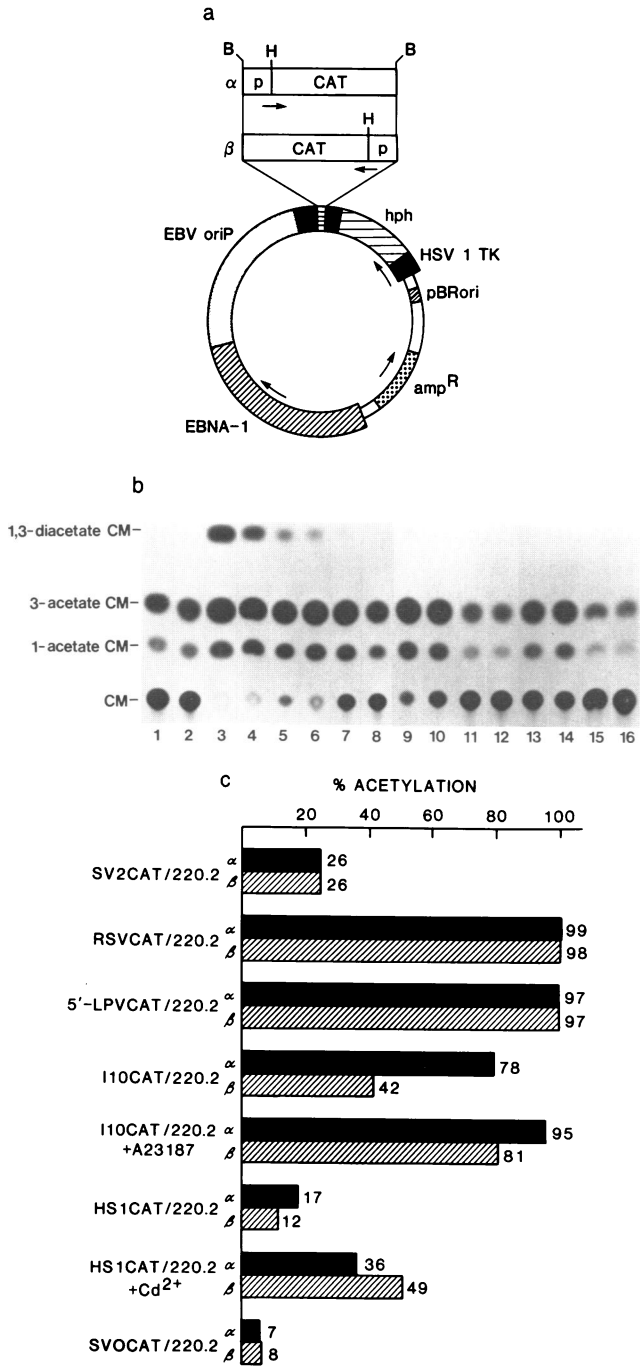

FIG. 2. Stable CAT expression in a human T-cell clone using different episome-based eukaryotic promoter elements. (*a*) Schematic representation of promoter–CAT/220.2 episomes, showing the EBV oriP, a functional segment of the EBV nuclear antigen 1 gene (EBNA-1), the *Escherichia coli hph* gene, herpes simplex virus thymidine kinase 1 (HSV 1 TK) promoter and termination sequences (solid bars), pUC12-derived multiple cloning site sequence (horizontal stripe), pBR322-derived sequences (narrow band), pBR322 origin of replication (pBRori), ampicillin-resistance gene (amp^R), and α- and β-oriented promoter–CAT cartridges. Arrows indicate direction of transcription. B, *Bam*HI; H, *Hin*dIII. (*b* and *c*) CAT activities for various promoter–CAT/220.2 V1 transfectants. TLC autoradiograms (*b*) and % acetylation (*c*) are shown. Numbers 1–16 in *b* correspond to promoter–CAT/220.2 constructs listed (top to bottom) in *c*, in that order. Numbers to right of bars (*c*) indicate % acetylation [1-acetylated + 3-acetylated + 1,3-diacetylated cpm/total (unacetylated + acetylated) cpm].

protocol described in *Results*, and transfectants were not recloned for these studies.

CAT Enzymatic Assay. Cells (5×10^6) were harvested, washed three times in PBS, resuspended in 25 mM Tris (pH 7.8) (100 μl) and lysed by five cycles of freeze–thawing. Crude cellular extracts (20 μl per reaction mixture) were assayed for chloramphenicol acetylating activity by a standard 1-hr assay as described (18). Enzymatic mixtures were extracted with ethyl acetate, and unacetylated and acetylated (1-acetate, 3-acetate, 1,3-diacetate) forms of [^{14}C]chloramphenicol were separated by ascending chloroform/methanol (95:5) thin-layer chromatography on silica gel plates (20 × 20 cm) (Whatman). Spots were visualized by overnight autoradiography (Kodak XAR film; −70°C with intensifying screen) and quantitated by excision of spots and liquid scintillation counting.

Flow Cytometry. Cells were immunostained as described (16) using OKT8 (anti-CD8), OKT3 (anti-CD3), OKT11 (anti-CD2; Ortho Diagnostics), or normal mouse IgG (Miles) as primary antibodies, and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Miles) was used as the secondary antibody. Cells were analyzed on an Epics V fluorescence-activated cell sorter (Coulter).

RESULTS

For these studies, we used the episomal replicon p220.2 (15), which contains the EBV sequences required for episomal replication in human cells, as well as the *E. coli hph* gene, which confers resistance to the eukaryocidal antibiotic hygromycin B. Since the human T-cell clones require feeder cells (PBMC) which are hygromycin B sensitive, we developed a modified selection protocol to permit the derivation of stable hygromycin B-resistant (hyg^R) transfectants. After transfection by electroporation or protoplast fusion, cells were resuspended in complete medium supplemented with IL-2. Cells were stimulated with IL-2, anti-CD3 antibody, and irradiated PBMC 2 days posttransfection, and hygromycin B was added 2 days later. A 7-day cycling scheme (Fig. 1) was then initiated in which hygromycin B was present in the culture medium only on days 2–7 after stimulation with irradiated fresh PBMC, IL-2, and anti-CD3 antibody. After 5 days of incubation with hygromycin B (0.15 mg/ml), control nontransfected cell cultures contained virtually no viable cells. Stable hyg^R transfectants have been reproducibly obtained (≈50% of transfections) for several cloned T-cell lines by this protocol.

A systematic investigation of promoter function in EBV episomal replicons has not been previously reported. To address this issue, as well as to specifically explore the utility of EBV-based episomal replicons for the expression of transfected genes in human T cells, we assessed episomal replicon-based promoter function in our cloned T-cell line V1. A panel of eukaryotic promoters consisting of various constitutive (simian virus 40 early, RSV 3′ LTR, LPV 5′ LTR) and inducible (*GRP78* gene, *hMTII$_A$* gene) promoters were examined. For these analyses, the prokaryotic CAT gene, which is absent in eukaryotic cells, served as a reporter gene for promoter-driven transcriptional activity (19, 26). Promoter–CAT cartridges were inserted, in both orientations, into the EBV-based replicon p220.2 at the unique *Bam*HI site, which lies just downstream of the EBV oriP (Fig. 2*a*; see *Materials and Methods*). The alternative orientations for cartridges in promoter–CAT/220.2 plasmids have been arbitrarily designated α (promoter is proximal to EBV oriP) and β (promoter is distal to EBV oriP).

Stable hyg^R V1 transfectants for each of the promoter–CAT/220.2 plasmids were independently derived, and the CAT enzymatic activity in them was compared (Fig. 2 *b* and *c*). The RSV 3′ LTR-based episomes yielded maximal CAT activity, which, based on titration experiments (data not shown), was marginally higher than the strong activity seen with the LPV 5′ LTR. In contrast, the episomes incorporating the simian virus 40 early promoter, a constitutive promoter commonly used in eukaryotic expression work (24), were significantly less efficient in driving CAT expression. The inducible GRP78 (I10CAT/220.2) and hMTII$_A$ (HS1CAT/220.2) gene promoters both displayed high levels of basal activity and showed 22% (7 μM A23187) and 93% (10 μM cadmium) induction for α-oriented promoters, respectively, and 112% and 308% induction for β-oriented promoters. However, the levels of CAT activity seen after induction of these promoters were significantly below those for either of the LTR-based (RSV and LPV) constitutive promoters. The orientation dependence of promoter activity ($\alpha > \beta$) seen with some promoters—e.g., the GRP78 gene promoter—may result from a transcriptional enhancer effect exerted by the EBV oriP (27). An episome analogous to RSVCATα/220.2, in which plus-sense CD8 instead of CAT is driven by the RSV 3′ LTR, yielded high levels of surface CD8 on transfectants (data not shown), establishing the utility of episome-based RSV 3′ LTR-driven expression systems for genes encoding cell-surface proteins as well as for cytoplasmic proteins.

To determine whether EBV episomal replicons can be used effectively for stable anti-sense RNA-mediated gene inhibition in cloned T cells, we selected CD8 as a model T-cell surface protein. To inhibit CD8 expression, we constructed the episome α-CD8/REP1 (Fig. 3). A 459-base-pair CD8 coding segment, spanning amino acids 9–161 of the 214-amino-acid-long processed CD8 protein, was first inserted in an inverted orientation downstream of the RSV 3′ LTR and upstream of the simian virus 40 polyadenylylation specification sequence from the late region of the virus. This promoter/α-CD8/polyadenylylation unit was subsequently cartridged in an α-orientation into the EBV episomal shuttle vector p220.2. α-CD8/REP1 was stably transfected into 8L2, a CD4$^-$CD8$^+$ T-cell clone derived by nonspecific stimulation via anti-CD3 mAbs and irradiated allogeneic feeder cells. The antigenic specificity and major histocompatibility complex restriction of this clone are unknown.

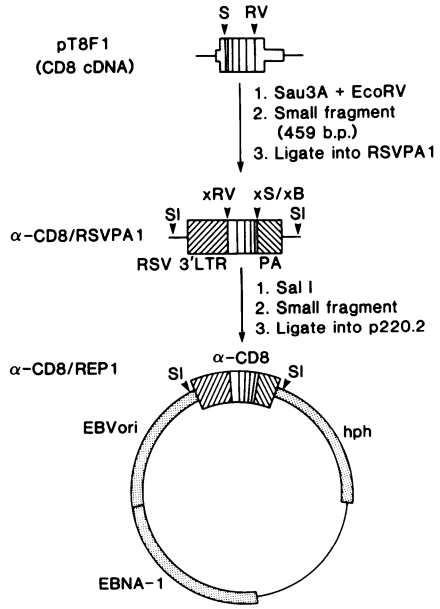

FIG. 3. Assembly of α-CD8/REP1 (anti-sense CD8) episome. Sources for pT8F1, p220.2, and RSVPA1 are described in *Materials and Methods*. S, *Sau*3A; RV, *Eco*RV; SI, *Sal* I. x indicates that the restriction site has been eliminated. PA, simian virus 40 late polyadenylylation/termination sequence; b.p., base pairs.

The level of surface CD8 expression on the parental and transfected 8L2 lines was determined 46 days posttransfection by flow cytometry using an anti-CD8 mAb (Fig. 4). Whereas nontransfected 8L2 and RSVCATα/220.2 8L2-transfected cells displayed equivalent levels of surface CD8, the α-CD8/REP1 8L2 transfectant demonstrated a marked decrease (>95%) in surface CD8 expression. The absence of alterations in the cell-surface expression of CD2 (sheep erythrocyte receptor) and CD3 (a component of the T-cell $\alpha\beta$ antigen receptor complex) in the α-CD8/REP1 transfectant (Fig. 4) established the specificity of this inhibition. Neoexpression of CD4 and CD1, surface molecules that are not expressed on 8L2 cells, was not observed for the CD8-suppressed transfectant (data not shown).

To establish that the anti-sense RNA inhibition was indeed mediated by an EBV-based episome, we transferred an aliquot of α-CD8/REP1-transfected cells into hygromycin B-free medium 39 days posttransfection and followed the stability of the mutant phenotype by serial flow cytometric analyses (Fig. 5). A gradual loss of EBV-based episomes is known to occur after removal of the selective agent (15). As expected, in the absence of hygromycin B, surface CD8 gradually reemerged in a time-dependent fashion, suggesting a progressive loss of the α-CD8/REP1 episome. After 1 week in hygromycin B-free medium, no change in the level of CD8 expression was detectable; after 2 weeks, an increase in CD8 expression was observed; by 5 weeks, surface CD8 levels approached those on the control RSVCATα/220.2 8L2 transfectants. In contrast, when the transfectant was maintained in the continuous presence of hygromycin B, CD8 inhibition remained stable for the entire 3-month observation period. The finding that the CD8-suppressed phenotype is stable for 1 week out of hygromycin B means that this agent can be temporarily cleared from the cells prior to functional assays. Also, the incremental increases in CD8 expression that occur after removal of the selective agent represent an experimental means for exploiting episome-based expression systems for gene dosage analyses.

Functional studies were performed on the α-CD8/REP1 transfectant grown in the continuous presence of hygromycin B. Nonspecific cytotoxicity mediated by either anti-CD3 mAb or the lectin phytohemagglutinin (PHA) did not differ significantly between the CD8 anti-sense mutant and controls (Fig. 6). Moreover, there were no significant differences in the expression of the IL-2 receptor (as detected by anti-Tac mAb) after anti-CD3 mAb stimulation, the proliferative response to either PHA or anti-CD mAb, or the response to phorbol diester as measured by increased IL-2 receptor expression (data not shown).

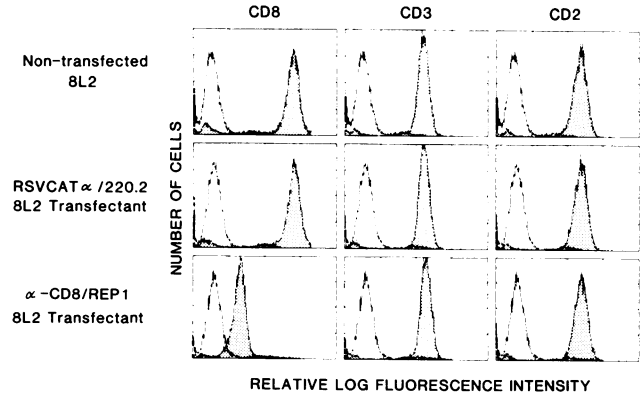

FIG. 4. Selective anti-sense RNA-mediated inhibition of surface CD8 expression on 8L2 cells. α-CD8/REP1 and RSVCATα/220.2 8L2 transfectants and parental 8L2 cells were stained on day 46 with OKT8 (anti-CD8), OKT3 (anti-CD3), and OKT11 (anti-CD2) (stippled areas) or normal mouse IgG (Miles; open areas) as primary antibodies, and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Miles) was used as the secondary antibody.

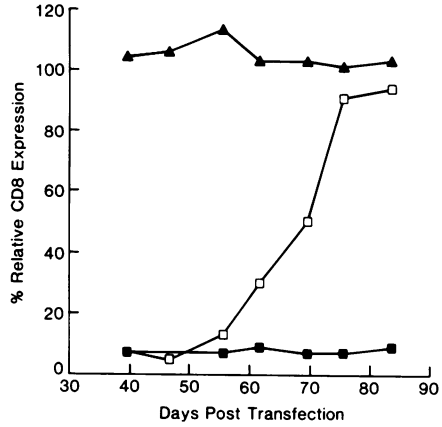

FIG. 5. Reappearance of surface CD8 on α-CD8/REP1 hygromycin B-resistant 8L2 transfectant line after removal of hygromycin B. Stable α-CD8/REP1 (squares) and RSVCATα/220.2 (triangles) 8L2 transfectants were derived as described in text. On day 39, a subculture of the α-CD8/REP1 transfectant (open squares) was washed free of hygromycin B and then maintained in the absence of this selective agent. CD8 expression was assessed at the indicated time points by flow cytometric analysis. Relative expression was calculated by comparing the peak channel number (pcn) for each group with the pcn for nontransfected 8L2 (pcn range, 190–201) at each time point.

DISCUSSION

In this study, we have described a methodology for the derivation and maintenance of stably transfected cloned human T lymphocytes. This entails the use of a 7-day cycling scheme in which the eukaryocidal antibiotic serving as a selective agent is absent for the first 2 days after the addition of irradiated antibiotic-sensitive feeder cells in each cycle.

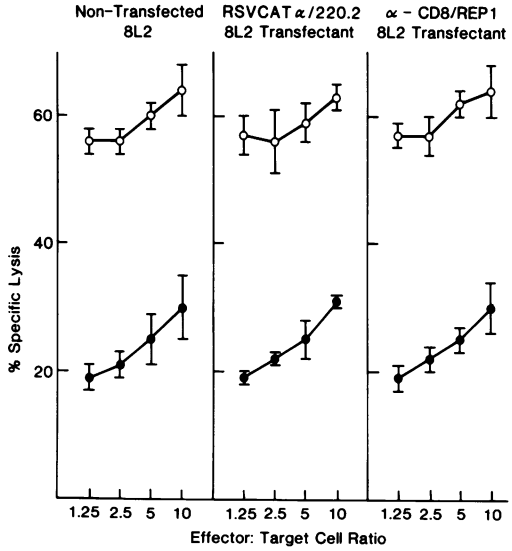

FIG. 6. Anti-CD3- and PHA-mediated cytotoxicity of U937 targets by nontransfected 8L2 cells and stable 8L2 transfectants (RSVCATα/220.2 and α-CD8/REP1). Cytolysis of ^{51}Cr-labeled U937 targets (5×10^3 cells per well) was mediated by either PHA (0.1 μg/ml) (open circles) or OKT3 (2 ng/ml) (solid circles) at various effector target cell ratios in a standard ^{51}Cr release assay (17). Maximal release of ^{51}Cr was achieved in 1% Triton X-100. Spontaneous release in these experiments was 16%. Specific lysis was calculated as described (17). Stable transfectants were selected in hygromycin B for 62 days and washed free of the antibiotic immediately prior to their use in this assay.

This protocol can be used in conjunction with electroporation or protoplast fusion as alternative transfection modalities and yields stable transfectants in a reproducible fashion.

Furthermore, we have shown that stable expression of transfected genes can be achieved in cloned human T cells by using an EBV-based episomal replicon. A comparison of five eukaryotic promoters demonstrated that the RSV 3′ LTR and LPV 5′ LTR are most suitable for high level EBV episome-based expression in T cells. Differences in CAT activity observed in this episomal expression system may reflect not only variation in promoter activity but also differences in episome copy numbers per cell, since it is conceivable that particular promoter sequence elements may influence EBV episomal replication capacity. EBV episomes have been reported to range up to 90 copies per cell (15). Although both inducible promoters studied here (*GRP78* and $hMTII_A$ genes) were inducible when episome-based and expressed in T cells, the fact that they demonstrated significant basal activity in the absence of their respective inducers could limit their usefulness for expression studies.

Using CD8 as a model T-cell-associated molecule, we have further demonstrated that episome-based, RSV 3′ LTR-driven expression systems can be used for anti-sense RNA-mediated gene inhibition. This approach can now be extended to anti-sense RNA work in other human hematopoietic and nonhematopoietic cell types. The gene inhibition achieved here was effective (>95% inhibition), selective (vis à vis CD3 and CD2), and reversible (by removal of the selective agent). Our results further indicated that the transfection/selection scheme did not interfere with cellular functions such as proliferation. Notably, we found no effect of inhibition of CD8, a marker for class I major histocompatibility complex-restricted cytotoxic T cells, on nonspecific cytotoxicity in our system. The possibility that the small amount of residual surface CD8 (<5%) in the α-CD8/REP1 transfectant was exerting a significant functional effect seems unlikely but cannot be definitively excluded. Since the antigenic specificity of this clone is unknown, studies of the effect of CD8 inhibition on antigen-mediated recognition await stable transfection of α-CD8/REP1 into a human T-cell clone with known antigenic specificity and major histocompatibility complex restriction.

In summary, these results establish the feasibility of deriving stable functionally intact anti-sense mutants of nontransformed human T-cell clones. Furthermore, this study demonstrates the utility of episomal expression vectors for achieving efficient anti-sense RNA-mediated gene inhibition in eukaryotic cells. An episome-based system not only offers an expeditious means for achieving amplification of transfected genes, but also permits the *in vitro* manipulation of transfected gene copy number by altering the selective pressure on the episomes. The selectivity of gene inhibition, with the possibility of confounding experimental artifacts minimized, is a distinct advantage of such an anti-sense RNA-mediated mutagenesis approach. In addition, successful transfection of human T-cell clones allows for the initiation of studies of functions that are associated with nontransformed cells such as regulated growth and antigen-specific cytotoxicity. The linking of gene-directed mutagenesis and T-cell cloning technologies should now permit a more precise definition of the functional roles of a variety of specific molecules in human T lymphocytes.

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