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We have found that genetic suppressor elements (GSEs) conferring drug resistance may function in combination rather than individually. To determine optimal conditions for the selection and analysis of GSE combinations, we have investigated parameters affecting the integration and gene expression from retroviral vector LNCX, used in the prior GSE studies. We have found that multicopy integration, which would allow combinatorial effects, is common even at intermediate infection rates, and that G418 selection for the *neo* gene of LNCX may result in inactivation of the unselected gene. We have generated a series of retroviral vectors carrying green fluorescent protein (GFP) rather than *neo* as a selectable marker and identified vectors that provide high GFP expression in MCF7 breast carcinoma cells. These vectors will be tested for optimal expression of the unselected reporter gene in MCF7. The most efficient vector will be used to clone a normalized population of cDNA fragments, which we have generated from the mRNA of MCF7 cells. To optimize phenotypic analysis of individual GSEs, we have introduced the *lac*I repressor into a subline of MCF7 cells and demonstrated regulated expression of a reporter gene from a β-galactoside regulated retroviral vector. We have also continued the analysis of GSE-mediated inhibition of the BCL2 gene in MCF7 cells.

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Introduction

Chemotherapy, based on cytotoxic drugs or hormone antagonists, is widely used as either adjuvant or primary treatment at all stages of breast cancer (1). The drug regimens that are most commonly used for breast cancer include a combination of cyclophosphamide, methotrexate and 5-fluorouracyl (CMF), or doxorubicin (presently the most powerful drug in this group) which is most often used as a single agent in the second line of treatment. In addition, taxol was shown by recent studies to produce significant objective responses in breast cancer. Aside from these chemotherapeutic compounds that are used in different types of cancer, another, breast cancer-specific class of drugs is used in the treatment of estrogen receptor-positive tumors. These agents are antiestrogens, the most widely used of which is tamoxifen (1). The objective of the present grant is to identify genes that determine the sensitivity of human breast carcinoma cells to agents used in breast cancer treatment, including antiestrogens (tamoxifen) and cytotoxic drugs (doxorubicin, cyclophosphamide, methotrexate, 5-fluorouracyl, taxol).

Our approach to the identification of chemotherapeutic sensitivity genes is based on the isolation of genetic suppressor elements (GSEs), derived from such genes and inducing cellular resistance or sensitivity to the corresponding agents. GSEs are short cDNA fragments that counteract the genes from which they are derived by encoding inhibitory peptides or antisense RNAs (2). We have previously developed the methodology for GSE selection from retroviral libraries carrying short random fragments of normalized (uniform-abundance) cDNA from mammalian cells (3-5). Using this approach, we identified several GSEs conferring resistance to anticancer drugs or inducing neoplastic transformation (4,5). The same strategy is being used in the present project to identify GSEs that render breast carcinoma cells resistant or sensitive to chemotherapeutic agents. In one arm of the project, GSEs inducing resistance to a metabolite of tamoxifen will be cloned by expression selection in MCF7 cells. The cloned GSEs will be used to isolate full-length cDNA sequences of the corresponding genes, and the effects of individual GSEs on hormone responsiveness and drug resistance will be investigated. In another arm of the project, different GSEs inducing resistance or sensitivity to chemotherapeutic drugs in various cell types, will be tested for their ability to make MCF7 cells resistant to cytotoxic drugs and antiestrogens. The genes giving rise to such GSEs would constitute likely determinants of chemotherapeutic sensitivity in breast carcinoma.

Body

1. Status of the project at the beginning of the year

During the first year of the project, we developed sublines of MCF7 breast carcinoma cells, transfected with the murine ecotropic receptor gene and thus

made susceptible to infection with ecotropic retroviruses. We have also tested the effect of a GSE isolated from the BCL2 apoptosis-suppressor gene on drug resistance in MCF7 cells and found that this GSE induced pronounced sensitization to several different drugs. In addition, we have initiated the selection of new GSEs conferring resistance to doxorubicin in MCF7 cells, using a normalized cDNA fragment library previously prepared from HeLa cells. Finally, we have synthesized cDNA from poly(A)+ RNA of MCF7 cells in preparation for cDNA normalization and construction of a normalized random fragment library in a retroviral vector. During the second year of the project, we have continued these studies, but some of our new results have necessitated additional effort aimed to improve the vector systems and selection strategies for GSE isolation and characterization. Furthermore, an unfortunate accident occurred in September 1995 when our laboratory moved to a newly constructed Molecular Biology Research Building. On the first weekend after the move, a power surge in the building disabled one of the freezers used to store cells in our laboratory. As the freezer alarm system was not yet set up in the building, this accident resulted in the loss of all the cells stored in the freezer, including the laboratory stock of MCF7 breast carcinoma cells and some of the MCF7 sublines developed in the first year of this project. Thus, some of the work had to be repeated, resulting in a setback.

2. Selection for GSEs conferring doxorubicin resistance.

In the previous period, we had initiated selection of GSEs conferring doxorubicin resistance in MCF7 cells. Cells transduced with a normalized HeLa cDNA fragment library in retroviral vector LNCX (6) yielded doxorubicin-resistant colonies at 4.5 times higher frequency than the control cells (transduced with insert-free LNCX vector), indicative of GSE selection. We have isolated individual doxorubicin-resistant lines and used PCR to recover and reclone integrated proviral inserts from these cells. A total of 15 clones were obtained, but MCF7 cells transduced with individual clones so far showed no significant increase in doxorubicin resistance relative to the control. Similar lack of efficacy of individual cloned elements enriched after drug selection was also observed in some other projects in our laboratory. Recently, however, we have obtained what is likely to be a general explanation for this problem: some GSEs induce a phenotype when introduced into recipient cells as a combination, but have little or no effect individually. This observation was first made in the selection of GSEs conferring resistance to aphidicolin in HT1080 cells. As illustrated in Fig. 1, resistance to aphidicolin (after 24, 48 or 72 hrs exposure) was observed in cells infected either with a second order library (a set of multiple sequences that were enriched after two rounds of aphidicolin selection) or with a clone combination 41/49, which contains three sequences that were enriched after three rounds of aphidicolin selection. In contrast, none of the individual sequences present in the 41/49 combination produced significant resistance to aphidicolin (data not shown). The same combinatorial effect was also obtained

in the selection of GSEs that induce neoplastic transformation of primate renal cells (data not shown). The finding that GSEs work in combinations has important consequences for all of our selection strategies. It implies that infection should be carried under the conditions where several retroviruses are integrated in most recipient cells, and that the integrated sequences should be recovered in the most comprehensive manner from the total population of selected cells, since a necessary component of an active combination may otherwise be lost. The original doxorubicin selection of MCF7 cells was not designed to satisfy these conditions and therefore will be repeated.

3. Analysis of parameters affecting the copy number and gene expression from retroviral vectors.

As indicated above, multicopy retroviral integration may be required for selecting and identifying active GSE combinations, but it is conventionally assumed that multicopy integration can only occur only under the conditions that result in 100% infection rate, or after multiple rounds of infection. These conditions were not present in our GSE studies, where active GSE combinations were nevertheless identified. We have conducted an extensive study aimed to determine the conditions for multicopy integration of retroviral vectors. Another goal of this study was to identify the cause for sporadic lack of GSE expression in cell populations infected with the GSE-carrying retrovirus. This analysis utilized HT1080 cell line, which is most commonly used in our drug-resistance GSE studies, and the retroviral vector LNCX (6), that we have predominantly utilized for GSE work in the past. This analysis has now been completed and submitted for publication (7); the manuscript has been provided in the Appendix. A brief summary of this study follows.

A subline of HT1080 cells, expressing the murine ecotropic receptor, was infected with LNCX retrovirus carrying the luciferase reporter gene and generated by transient transfection of BOSC 23 packaging cells. Mass populations of cells infected under conditions resulting in different initial infection rates (IIR) and selected with G418, showed highly variable luciferase activity. Luciferase expression in cell populations with IIR≤5% was generally low; many populations with IIR<1% had marginal or no luciferase activity. The loss of luciferase expression in low-IIR populations was associated with G418 selection. In contrast, cell populations with IIR≥6% showed higher luciferase expression, which was strongly correlated with the IIR. Southern hybridization analysis showed that most cells of the low-IIR populations carried one integrated provirus, with a high incidence of structural rearrangements that abolished luciferase activity. Surprisingly, all the populations with IIR≥6% contained two or more copies of integrated provirus per cell, and their luciferase activity correlated with the provirus copy number. Luciferase expression was relatively stable in the populations with IIR>1% maintained in the absence of G418. Increasing the selective concentration of G418 or prolonged maintenance of cell populations in the presence of G418 resulted in higher incidence of provirus

rearrangements and decreased luciferase expression. These results indicate that (i) G418 selection after infection at low IIR can result in inactivation of the unselected gene or GSE and (ii) co-integration of multiple proviruses is common under conditions of intermediate infection rate, thus accounting for the selection of GSE combinations in our studies.

4. Development of retroviral vectors carrying Green Fluorescent Protein as a selectable marker and selection of optimal vectors for GSE expression in MCF7 cells.

One of the conclusions of the above described study is that the use of prolonged G418 selection for infected cells is fraught with a risk of selecting for proviruses in which the unselected gene or GSE has been inactivated. Thus, we felt that the LNCX vector that carries the *neo* gene as a selectable marker should be replaced in the future GSE selections, in particular in the tamoxifen selection which will be conducted in the present project. A particularly attractive selectable marker is the Green Fluorescent Protein (GFP), originally isolated from fish Aequorea victoria (8). Cells expressing this protein can be isolated through a non-toxic process of fluorescence-activated cell sorting, very shortly after gene transfer. In contrast to drug selection, continuous expression of this marker would not be required after sorting, thus eliminating the selective pressure against the gene of interest. We have decided therefore to develop retroviral vectors carrying GFP as the selectable marker. The originally isolated GFP gene (8), however, did not provide us with significant fluorescence in mammalian cells. More recently, however, two modified versions of the GFP gene have become commercially available. The first version is the Red-shifted GFP (RFP), the amino acid sequence of which has been changed (S65T substitution) to increase the fluorescence intensity and the emission wave length, which matches closely with that of FITC, a commonly used fluorescent tag (9). The second version is the Green Lantern (GL) gene, which also encodes RFP but is translated much more efficiently due to optimization of its codon usage for mammalian cells (10). We have constructed a series of retroviral vectors, with different promoters driving the expression of the RFP and, subsequently, of the GL gene. The different constructs that we have generated are shown in Fig. 2. The activity of these constructs has been tested in several cell lines, including MCF7. Fig. 3 shows flow profiles of MCF7 cells (carrying ecotropic receptor) that were either uninfected or infected with the vectors LXSG or LGCX. Cells transduced with these vectors appear highly fluorescent, indicating high efficiency of GFP expression and the ability to isolate the infected MCF7 cells by flow sorting. We have also observed (data not shown) that the fluorescence intensity of cells transduced with the same GFP vector is positively correlated with the infection rate. In light of our previous study (7; see section 3), this result can be interpreted as indicative of elevated copy number of integrated retroviruses in cells infected under high-IIR conditions. This indicates that sorting for cells with the highest level of GFP fluorescence would yield a

population of multicopy-infected cells, which would be particularly suitable for the selection and testing of GSE combinations. Furthermore, no significant loss of GFP fluorescence has been observed in MCF7 cells maintained in culture for 3 weeks after transduction (data not shown), indicating the stability of these vectors in MCF7 cells.

In addition to identifying the vectors that provide for the optimal expression of the selectable marker in MCF7 cells, we are also analyzing the efficacy of expression of the unselected genes from such vectors, using luciferase as the reporter gene. Thus, we have found that luciferase expression in MCF7 cells transduced with the LNCX vector is slightly higher than the expression from the vector LXSN (data not shown). We have also inserted the luciferase reporter gene into the cloning sites of several GFP-carrying vectors. The efficacy of luciferase expression from these vectors will be determined after transduction into MCF7 cells and isolation of the infectants by flow sorting. On the basis of these assays, we will select the vector that will be used to construct a normalized cDNA library from MCF7 RNA and to test different GSEs in this cell line.

5. β-galactoside regulated gene expression in MCF7 cells.

Another approach to overcoming the problems of variability of GSE expression in retrovirus-transduced cells is to use a regulated gene expression system, where expression of the gene of interest could be activated by a physiologically neutral inducer. In a separate study (11), we have developed a set of three retroviral vectors, carrying different promoters coupled with bacterial *lac* operator sequences. In cells expressing the bacterial *lac* repressor, these promoters are regulated by β-galactosides, IPTG or MTG. To enable regulated expression of these promoters in MCF7 cells, we have transfected these cells with a plasmid p3'SS (Stratagene) expressing a modified *lacI* repressor. We then screened individual transfectants for the ability to show regulated expression of a luciferase reporter gene. The best regulation was observed in a cell line designated MCF7/lac. In the experiment shown in Fig. 4, this line was infected with the LNXRO2 vector (11) carrying the luciferase gene under a lacI-regulated promoter. After G418 selection of infectants, luciferase expression was measured in the presence and in the absence of 5 mM IPTG. The presence of IPTG resulted in 28-fold higher luciferase expression in the MCF7/lac cells. In contrast, MCF7 cells without *lacI* repressor showed high luciferase expression both in the presence and in the absence of the inducer. We are planning to use the MCF7/lac cell line for future testing of the effects of different GSEs on drug resistance of breast carcinoma cells.

6. Effects of BCL2-derived GSE on MCF7 cells.

As described in the previous report, we have observed (in multiple independent experiments) that the expression of a sense-oriented 81 bp fragment of BCL2 cDNA (termed 2-7), selected for the ability to potentiate apoptosis, sensitized

MCF7 cells to several different drugs. This sensitization was associated with a striking decrease in the amount of BCL2 RNA expressed in the cells. In contrast, the decrease in the steady-state level of the BCL2 protein appeared to be much less drastic than the decrease in its RNA. To determine if the 2-7 GSE promoted the degradation of an untranslated cytoplasmic pool of BCL2 mRNA, we compared the representation of BCL2 mRNA in the total cellular, nuclear, polysomal and cytoplasmic non-polysomal (free RNP) RNA fractions extracted from the GSE-transduced and the control (LNCX vector-transduced) cells. The RT-PCR analysis in Fig. 5 showed, however, that the loss of BCL2 sequences was equally pronounced in all the RNA fractions from 2-7 transduced cells, arguing against selective degradation of untranslated RNA.

The above result was most consistent with the possibility that the 2-7 GSE inhibited BCL2 expression at the level of transcription. Other investigators have demonstrated that BCL2 transcription in MCF7 cells is positively regulated by estrogen. Though all of our experiments were carried out in estrogen-containing media (with phenol red), we wanted to determine if the effects of the 2-7 GSE could be mediated through changes in the cellular levels of the estrogen receptor (ER). Western blot analysis of ER in cell populations transduced with the LNCX vector, the 2-7 GSE, its mutant forms (2-25 and 2-25Δ) or with the antisense BCL2 RNA is shown in Fig. 6. This analysis indicated that the loss of BCL2 mRNA (which occurred in cells transduced with 2-7, 2-25 and 2-25Δ) was not associated with major changes in the ER level.

Up to this point, all the studies of BCL2-GSE effects on MCF7 cells had been done on a stock of MCF7 cells that was established in our laboratory and that expressed a high level of BCL2 mRNA. Unfortunately, this MCF7 stock was lost in the freezer accident. While two sublines established earlier from this stock by transfection with the murine ecotropic receptor (ETR) were still available, these sublines were found to have lost their BCL2 expression (we have observed that spontaneous loss of BCL2 mRNA could occur when cells were allowed to grow to a high density; low cell density was therefore meticulously maintained in all the experiments with BCL2-GSE). We have therefore obtained a new vial of MCF7 cells from ATCC. These cells were found to express BCL2 mRNA, though at a slightly lower level than the cells that we originally used. However, when we introduced the BCL2-GSE into these cells, we saw no decrease in either BCL2 mRNA levels or in cellular drug resistance (data not shown). A potential explanation for this discrepancy has been suggested, however, by a recent experiment (Fig. 7). In this experiment, we have compared the levels of estrogen receptor (ER) between the ATCC stock of MCF7 cells and the two ETR-expressing sublines derived from our laboratory stock. To our surprise, the cells from ATCC showed a much higher level of ER than our cell lines (Fig. 7). We hypothesize that the loss of ER could have occurred at an early stage in the establishment of our laboratory stock of MCF7 cells. If the ER levels were low in our MCF7 stock used for BCL2-GSE studies, the high-level of BCL2 expression could be due to some estrogen-unrelated regulatory factors, that were susceptible to the effect of the BCL2-GSE. In

contrast, BCL2 expression in the ATCC stock of MCF7 cells was maintained by positive estrogen regulation through the high levels of ER in these cells, and was therefore insensitive to the GSE inhibition. We are planning to test this hypothesis by modulating the estrogen levels in the GSE-transduced and control cells and by introducing an ER-overexpressing construct into the cells with a low ER expression.

The low levels of ER in our ETR-transfected sublines raises another issue, since we had been planning to use these sublines for tamoxifen selection. While these lines are still susceptible to tamoxifen, we are planning to determine if the ATCC stock of MCF7 cells, expressing high levels of ER, would be more sensitive to this drug. It may be advantageous to carry out parallel selection of tamoxifen-resistance GSEs in both high-ER and low-ER sublines, as different mechanisms of resistance are likely to be selected in these two types of cells.

7. Preparation of a population of normalized cDNA fragments from MCF-7 cells

To obtain a normalized (uniform-abundance) population of the cDNA fragments from MCF7 cells, we have carried out Cot fractionation of adapter-tagged cDNA fragments prepared in the previous period of the project. Hydroxyapatite fractionation of DNA samples reannealed for different periods of time was carried out as previously described (4,12), and individual fractions were amplified by PCR and characterized by Southern hybridization with probes corresponding to the highly-expressed tubulin and 28S RNA, intermediately expressed C-FOS and low-level expressed MDR1 genes. As illustrated in Fig. 8, the reannealing results in decreased representation of highly-expressed genes and increased representation of weakly-expressed genes in the single-stranded DNA fractions. The best-matched representation of different cDNA sequences is observed in the fraction derived from the DNA that remained single-stranded after 48 hrs of annealing. This fraction will be used for cloning into a GFP-carrying vector that will show the best expression in MCF7 cells (see section 4 above).

Conclusions

The results obtained in the past year of the project have indicated the need to modify the procedures for GSE selection and testing in such a way as to maximize the probability of co-integration of different retroviruses and to minimize the use of G418 selection for the infected cells. Two classes of retroviral vectors developed in this period address these requirements and have been found to be usable in MCF7 breast carcinoma cells. The first set of vectors contains GFP as the selectable marker, making it possible to isolate infected cells without prolonged drug selection and to isolate the cells carrying multiple retroviruses. The second set of vectors, which are regulated by β-galactosides in MCF7 cells expressing the *lacI* repressor, is uniquely useful for studying phenotypic effects of individual GSEs in a tightly controlled manner, at the level

of either cell populations or individual clones. The use of these vectors should provide a major improvement for GSE selection and analysis.

The observed spontaneous changes in the estrogen receptor levels in different sublines of MCF7 cells indicate the need to monitor these levels prior to and during tamoxifen selection. These changes also provide a testable hypothesis explaining the differences in the response to the BCL2-derived GSE in different subpopulations of MCF7 cells.

In the next period of the project, we are planning to identify the most suitable vector for GSE selection in MCF7 cells and use this vector to clone the normalized population of MCF7 cDNA fragments that we have generated. We will also develop a subline of MCF7 cells which would be highly infectable with ecotropic retrovirus and, at the same time, would maintain a high level of estrogen receptor. This cell/library combination will be used to select GSEs conferring tamoxifen resistance. We will also repeat doxorubicin selection, using the HeLa-derived library, under the conditions that would favor multicopy integration and comprehensive insert recovery from the selected cells. Finally, we will test the hypothesis that the BCL2-derived 2-7 GSE specifically affects estrogen-independent BCL2 transcription and will investigate the mechanism of this regulation.

References

*submitted in the Appendix

1. Harris, J.R., Morrow, M., and Bonadonna, G. (1993). Cancer of the breast. In: *Cancer: Principles and Practice of Oncology*, 4th Edition (DeVita, V.T., Hellman, S. and Rosenberg, S.A., eds.), Philadelphia: Lippincott, pp. 1264-1332.
2. Holzmayer, T.A., Pestov, D.G., and Roninson, I.B. (1992). Isolation of dominant negative mutants and inhibitory antisense RNA sequences by expression selection of random DNA fragments. Nucleic Acids Res. 20, 711-717.
3. Gudkov, A.V., Zelnick, C., Kazarov, A.R., Thimmapaya, R., Suttle, D.P., Beck, W.T., and Roninson, I.B. (1993). Isolation of genetic suppressor elements, inducing resistance to topoisomerase II-interactive cytotoxic drugs, from human topoisomerase II cDNA. Proc. Natl. Acad. Sci. USA 90, 3231-3235.
4. Gudkov, A.V., Kazarov, A.R., Thimmapaya, R., Axenovich, S., Mazo, I., and Roninson, I.B. (1994). Cloning mammalian genes by expression selection of genetic suppressor elements: association of kinesin with drug resistance and cell immortalization. Proc. Natl. Acad. Sci. USA, in press.
5. Roninson, I.B., Gudkov, A.V., Holzmayer, T.A., Kirschling, D.J., Kazarov, Kazarov, A.R., Zelnick, C.R., Mazo, I.A., Axenovich, S., and Thimmapaya, R.

(1995). Genetic suppressor elements: new tools for molecular oncology. *Cancer Res.* 55, 4023-4028.

6. Miller, A.D. and Rosman, G.J. (1989). Improved retroviral vectors for gene transfer and expression. Biotechniques 7, 980-986.

*7. Schott, B., Iraj, E.S., and Roninson, I.B. (1996). Effects of infection rate and selection pressure on gene expression from an internal promoter of a double-gene retroviral vector. Submitted for publication.

8. Chalfie M., Tu Y., Euskirchen G., Ward W. W. and Prasher D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* 263, 802-805.

9. Heim, R., Cubitt, A.B., and Tsien, R.Y. (1995). Improved green fluorescent protein. Nature 373, 663-664.

10. Zolotukhin, S., Potter, M., Hauswirth, W.M., Guy, J., and Muzyczka, N. (1996). A "humanized" green fluorescent protein cDNA adapted for high-level expression in mammalian cells. J. Virol. 70, 4646-4654.

11. Chang, B.D. and Roninson, I.B. (1996). Inducible retroviral vectors regulated by *lac* repressor in mammalian cells. *Gene*, in press.

12. Gudkov, A.V. and Roninson, I.B. (1996). Isolation of genetic suppressor elements (GSEs) from random fragment cDNA libraries in retroviral vectors. In: *Protocols for cDNA libraries*, I.G. Cowell and C.A. Austin, Eds., Humana Press, London, in press.

13. Noonan, K.E., Beck, C., Holzmayer, T. A., Chin, J.E., Wunder, J.S., Andrulis, I.L., Gazdar, A.F., Willman, C.L., Griffith, B., Von Hoff, D.D., and Roninson, I.B. (1990). Quantitative analysis of *MDR*1 (multidrug resistance) gene expression in human tumors by polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* 87, 7160-7164.

Appendix

Figure Legends

Figure 1. Aphidicolin resistance of HT1080 cells transduced with GSE combinations. HT1080 cells (with ecotropic receptor) were transduced with insert-free vector (LNCX), a mixture of clones carrying three different sequences enriched by three rounds of aphidicolin selection (Clns 41/49), or with a sublibrary derived after 2 rounds of selection (2d order library). Cells were plated in 6-well plates in duplicates (10^4 cells for drug treatment or $5x10^2$ cells for plating efficiency control [PE]). After treatment with 4 μg/ml aphidicolin for the indicated periods of time, cells were allowed to form colonies. The experimental and PE control plates were stained with methylene blue, after which the dye was extracted in 1N HCl and OD_{680} was determined. "Growth in aphidicolin" represents the ratio of OD for aphidicolin-treated and PE control samples.

Figure 2. Diagrammatic representation of GFP-expressing retroviral vectors. Vectors are shown in the form of integrated proviruses. Vector elements are described underneath the figure.

Figure 3. Fluorescence profiles of MCF7 cells transduced with GFP-expressing retroviral vectors. MCF7 cells (carrying the ecotropic receptor) were either uninfected (A) or infected with the vectors LXSG (B) or LXCR (C). 2 days after infection, cell fluorescence was analyzed using FACSort (Becton-Dickinson).

Figure 4. Regulated expression of luciferase in MCF7 cells expressing *lacI* repressor. Subline MCF7/lac, expressing lacI repressor, as well as unmodified MCF7 cells, were infected with retroviral vector LNXR02 (11) carrying the firefly luciferase gene, and infectants were selected with G418. For luciferase assays, cells were incubated for 24 hrs in the presence or absence of 10 mM IPTG. Luciferase activity was measured using Luciferase Assay System (Promega) and normalized for the amount of cellular protein in each sample (as determined using a Bio-Rad Protein Assay Reagent).

Figure 5. Loss of BCL2 sequences from different RNA fractions of MCF7 cells transduced with BCL2-derived 2-7 GSE. MCF7 cells were infected either with the LNCX vector or with LNCX carrying the BCL2-derived 2-7 GSE, and infected cell populations were selected with G418. RNA was extracted from total cells, from the purified nuclei, or from the cytoplasmic fraction which was further separated by sucrose step gradient centrifugation into polysomal and "free" RNP fractions. Expression of the BCL2 gene was analyzed by RT-PCR, using β_2-microglobulin as an internal control (13).

Figure 6. Western blot analysis of estrogen receptor in MCF7 cell populations transduced with BCL2-related constructs. MCF7 cells (laboratory stock) were

infected with the retroviral vectors LNCX, LNCX with BCL2-derived 2-7 GSE, mutant versions of 2-7 (2-25 and 2-25Δ), or LNCX with antisense-oriented BCL2 cDNA, and selected with G418. A mouse mAb, GR17 (Oncogene Science) against human estrogen receptor, was used for Western blot analysis, carried out under the manufacturer-supplied protocol. Proteins were visualized by autoradiography after incubation with peroxidase-conjugated second antibody (Caltag) and chemiluminescent substrate (Kirkegaard & Perry Laboratories).

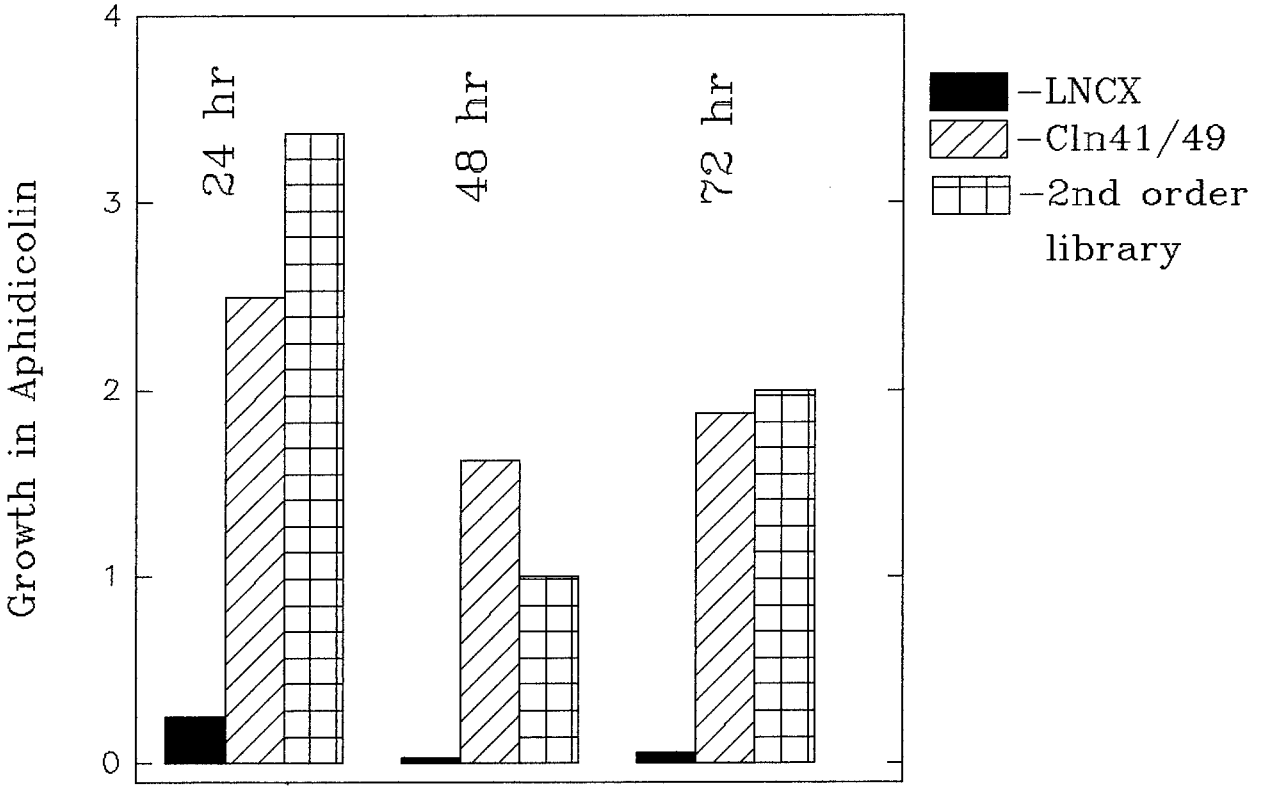
Figure 7. Western blot analysis of estrogen receptor in MCF7 cells from ATCC and in two laboratory sublines. MCF7 cells from ATCC, growing in the indicated types of sera, and clonal lines #2 and #7, derived from the laboratory stock of MCF7 after transfection with a vector expressing the ecotropic receptor, were analyzed for estrogen receptor expression, as described in the legend to Fig. 6.

Figure 8. Analysis of Cot fractionated cDNA fractions from MCF7 cells by Southern hybridization with probes for genes expressed at different levels. The origin of the cDNA fraction in each lane is indicated under the figure. Each cDNA fraction was amplified by PCR using adaptor-derived primers. Equal amounts of each PCR product were separated by electrophoresis in 2% agarose gel and analyzed by Southern hybridization with cloned cDNA probes for the indicated human genes.

Manuscript attached

Schott, B., Iraj, E.S., and Roninson, I.B. (1996). Effects of infection rate and selection pressure on gene expression from an internal promoter of a double-gene retroviral vector. Submitted for publication.

FIGURE 1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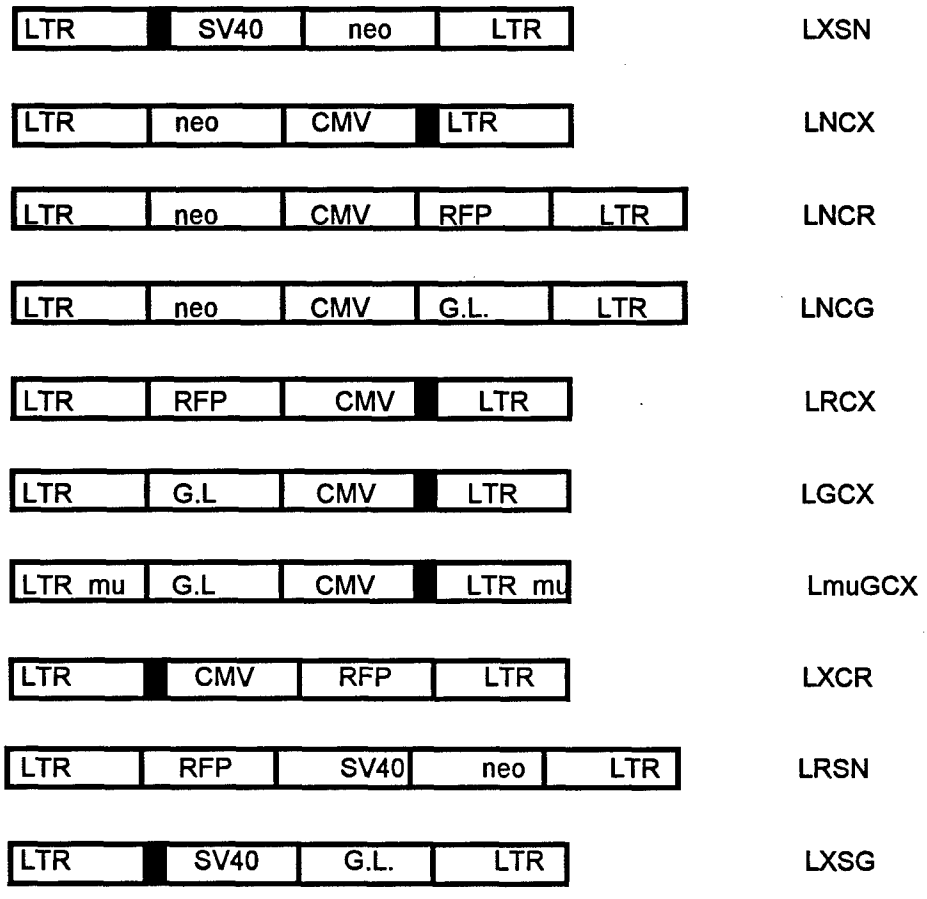
72 hr
-LNCX
-Cln41/49
-2nd order library

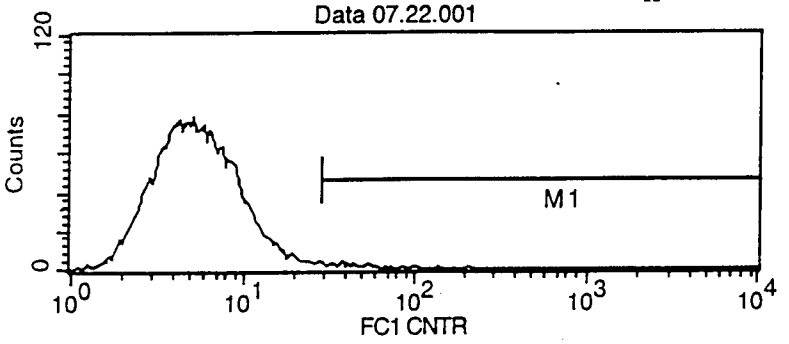
Figure 2



Legend:
G.L. - Green Lantern variant of green fluorescent protein.
RFP - Red-shifted variant (S65T mutation) of green fluorescent protein.
LTR - Long terminal repeat from MoMu retrovirus.
LTR mu - Long terminal repeat from MoMu retrovirus with an extra Sp1 site.
SV40 - SV40 immediate early promoter and enhancer.
CMV - Human cytomegalovirus immediate early promoter and enhancer.
neo - Neomycin phosphotransferase, conferes neomycin (G418) resistance.
▮ - Cloning sites.

FIGURE 3

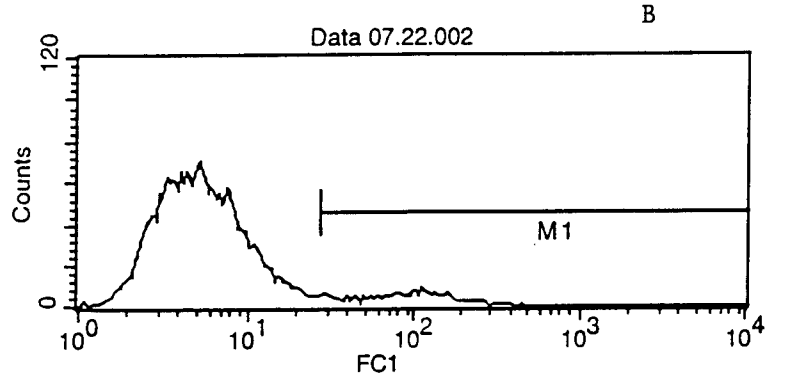
A



File: Data 07.22.001 Acquisition Date: 2:
Gate: G3 Gated Events: 109
Total Events: 12913 X Parameter: FL1-H

Marker	Events	% Gated	% Total	Mean	
All	10947	100.00	84.78	6.92	17
M1	137	1.25	1.06	74.64	1(

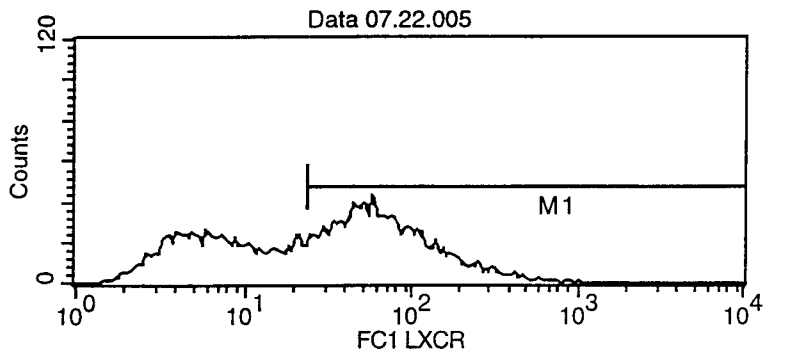
B



File: Data 07.22.002 Acquisition Date: 2
Gate: G3 Gated Events: 10
Total Events: 12547 X Parameter: FL1-H

Marker	Events	% Gated	% Total	Mean	
All	10862	100.00	86.57	14.58	2
M1	894	8.23	7.13	106.93	

C



File: Data 07.22.005 Acquisition Date: 2
Gate: G3 Gated Events: 10
Total Events: 12390 X Parameter: FL1-H

Marker	Events	% Gated	% Total	Mean	
All	10642	100.00	85.89	56.33	1
M1	6088	57.21	49.14	91.91	1

FIGURE 4

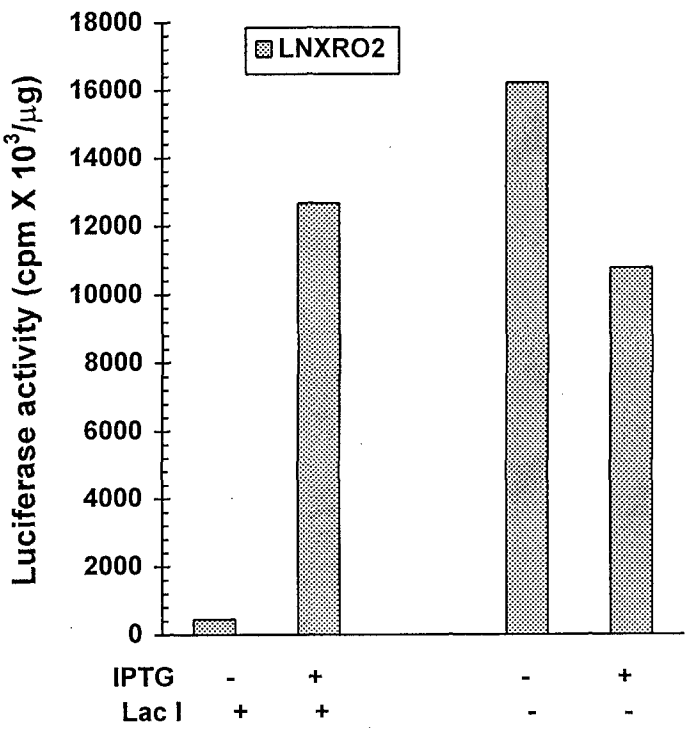
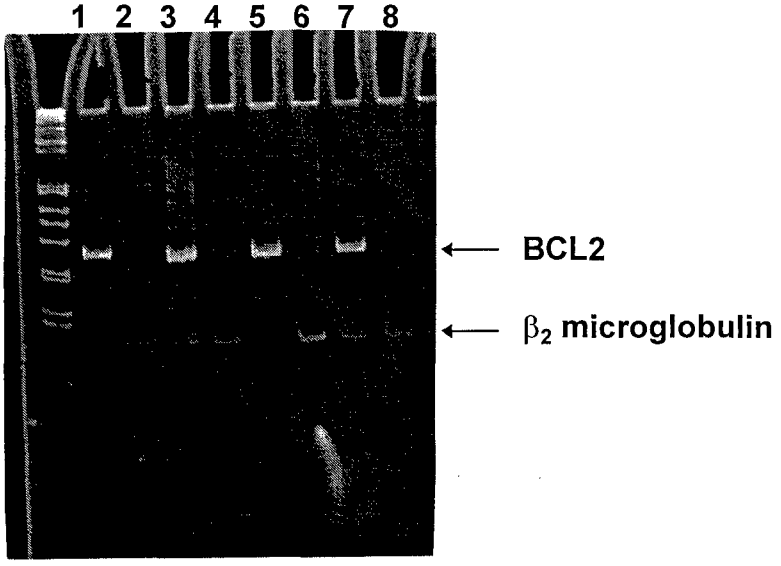

LNXRO2
Luciferase activity (cpm X 10³/µg)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
IPTG - + - +
Lac I + + - -

FIGURE 5



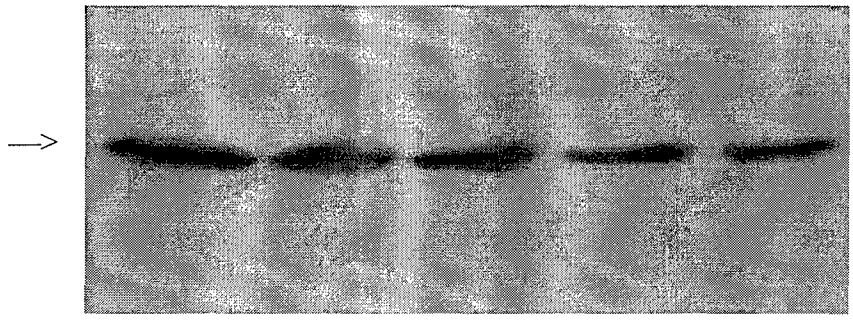
Lane 1. Total RNA, MCF7 with LNCX
Lane 2. Total RNA, MCF7 with LNC2-7

Lane 3. Nuclear RNA, MCF7 with LNCX
Lane 4. Nuclear RNA, MCF7 with LNC2-7

Lane 5. Polysomal RNA, MCF7 with LNCX
Lane 6. Polysomal RNA, MCF7 with LNC2-7

Lane 7. "Free" RNP, MCF7 with LNCX
Lane 8. "Free" RNP, MCF7 with LNC2-7

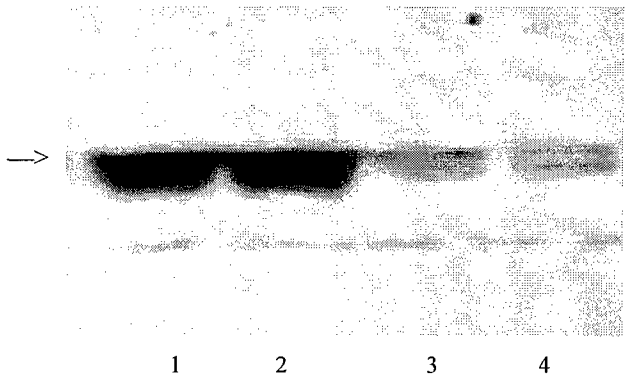
FIGURE 6



1 2 3 4 5

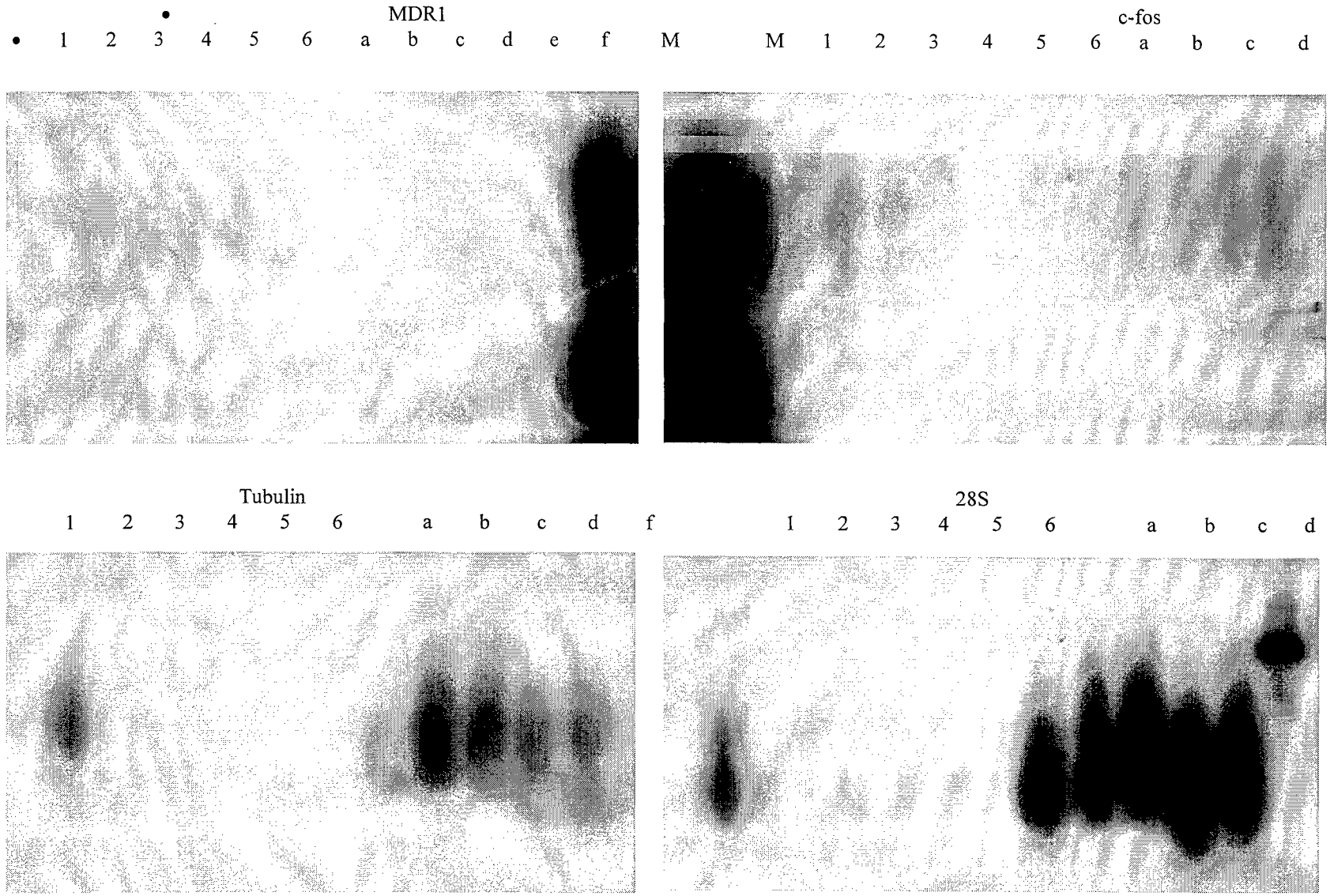
Lane 1: LNCX control; Lane 2: 2-25; Lane 3: 2-25Δ; Lane 4: 2-7;
Lane 5: BCL2 antisense. Each lane is loaded with 50 μg of protein.

FIGURE 7



Lane1-MCF7 in FBS; Lane2-MCF7 in FCI; Lane3-Clone #2; Lane4-Clone #7. Each lane is loaded with 100 ug of protein.

FIGURE 8



Lane 1-6, single-stranded DNA; Lane a-f, double-stranded DNA; Lane 1 and a- 0 hour; Lane 2 and b- 12 hrs; Lane 3 and c- 24 hrs; Lane 4 and d- 48 hrs; Lane 5 and f- 72 hrs; Lane 6 and f- 96 hrs; M- 1 Kb DNA ladders.

Effects of infection rate and selection pressure on gene expression from an internal promoter of a double gene retroviral vector

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Running title: Gene expression from retroviral vector

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ABSTRACT

Many commonly used retroviral vectors express one gene from the viral long terminal repeat (LTR) promoter and another gene from an internal promoter. We have investigated factors affecting the expression of the luciferase reporter gene from the internal cytomegalovirus-derived promoter of the retroviral vector, LNCX, which contains a LTR-driven *neo* gene as a selectable marker. A subline of human HT1080 cells, expressing the murine ecotropic receptor, was infected with retrovirus generated by transient transfection of BOSC 23 packaging cells. Mass populations of cells infected under conditions resulting in different initial infection rates (IIR) and selected with G418, showed highly variable luciferase activity. Luciferase expression in cell populations with IIR≤5% was generally low; many populations with IIR<1% had marginal or no luciferase activity. The loss of luciferase expression in low-IIR populations was associated with G418 selection. In contrast, cell populations with IIR≥6% showed higher luciferase expression, which was strongly correlated with the IIR. Southern hybridization analysis showed that most cells of the low-IIR populations carried one integrated provirus, with a high incidence of structural rearrangements that abolished luciferase activity. In contrast, populations with IIR≥6% contained two or more copies of integrated provirus per cell, and their luciferase activity correlated with the provirus copy number. Luciferase expression was relatively stable in the populations with IIR>1% maintained in the absence of G418. Increasing the selective concentration of G418 or prolonged maintenance of cell populations in the presence of G418 resulted in higher incidence of provirus rearrangements and decreased luciferase

expression. These results indicate that the negative effect of selection for the LTR-driven gene on gene expression from an internal promoter depends on the selection stringency and can be obviated by increasing the infection rate.

INTRODUCTION

Retroviral transduction provides one of the most efficient means for stable gene transfer in a wide variety of mammalian cell types, both *in vitro* and *in vivo*. It is also the easiest of all the virus-based transduction techniques, which was recently simplified by the development of improved transient transfection procedures for generating high titers of recombinant retroviruses (1, 2). The high efficiency of retroviral transduction allows one to analyze phenotypic effects of transduced genes in mass populations rather than individual clones of infected cells, thus avoiding the problems associated with clonal variability. Since it is not always feasible to infect 100% of the recipient cells, population assays usually utilize double-gene vectors that express a dominant selectable marker in addition to the gene of interest. Such markers allow one to select a population consisting entirely of infected cells, regardless of the infection rate. The most commonly used retroviral vectors express one gene from the viral long terminal repeat (LTR) promoter and the second gene from an internal promoter (in either sense or antisense orientation relative to LTR), though single-promoter bicistronic vectors containing an internal ribosome entry site (IRES) have gained popularity in recent years (3; 4). The use of an internal promoter, rather than LTR, becomes particularly important when such a promoter provides cell-type specific or inducible expression.

It has long been known that the two promoters of a double-gene retroviral vector are not always coexpressed, and that functional selection for one promoter can lead to inactivation of the other (5). Another well-known problem, which is not limited to double-gene vectors, is the instability of gene expression from integrated proviruses (6, 7). A

number of studies have addressed the parameters that affect the level or the stability of expression from retroviral vectors (7-16). Several important factors have been identified, including the nature of the recipient cells, relative strength and orientation of the promoters, virus titer, provirus copy number and integration sites, and maintenance of selective pressure. The role for some of these factors, however, remains controversial, with different correlations reported for different cell/vector systems. For example, maintaining the selection pressure was reported in some studies to increase (7) and in other studies to decrease (12) the stability of expression of the nonselected gene. In several studies, the presence of more than one copy of integrated provirus per cell was correlated with higher expression levels and stability (11,12). Multicopy integration in infected cell populations has been achieved through multiple rounds of infection or by using very high virus titers (12,14,17).

Our laboratory has extensively utilized double-gene retroviral vectors carrying the *neo* (G418 resistance) gene to analyze phenotypic effects of different genetic elements in mass populations of infected cells (17-19). In some cases, however, we encountered difficulties in reproducing the same phenotypic effect in different independently derived populations. Experimental variability was primarily observed in the experiments where the infection rates were low and G418 selection was carried out prior to phenotypic analysis. In the present study, we have investigated the effect of differences in the infection rate or in the conditions of G418 selection on the expression of a reporter gene from an internal cytomegalovirus (CMV)-derived promoter of a commonly used double-gene retroviral vector, LNCX (20).

MATERIALS AND METHODS

Vectors and cell lines. The Moloney virus-based retroviral vector LNCX (20) was a gift of A.D. Miller (Fred Hutchison Cancer Center). LNCluc was constructed by B.-d. Chang in our laboratory by blunt-end ligation of the 1.7 kb firefly luciferase gene, excised with StuI from pGEMluc vector (Promega), into the unique HpaI site located downstream of the CMV promoter in the LNCX vector. BOSC 23 ecotropic packaging cell line, a derivative of human 293 cells (1), and their amphotropic counterpart, BING (21), were a gift of W.S. Pear and D. Baltimore (MIT). PE501 and PA317, murine ecotropic and amphotropic packaging cells (20, 22), were a gift of A.D. Miller. HT1080 pJet-2fTGH cell line (kindly provided by G.R. Stark, Cleveland Clinic Research Foundation) was generated by cotransfection of HT1080 2fTGH line (23) with pJET plasmid expressing the murine ecotropic receptor (24) and a plasmid expressing the puromycin-resistance gene. The original HT1080 cells (without ecotropic receptor) and NIH 3T3 cells were obtained from ATCC. All cells were grown at 37°C in a 7% CO_2 atmosphere, in DMEM supplemented with 10% fetal calf serum and with penicillin-streptomycin.

Retroviral infection and isolation of infected cell populations and clones. BOSC 23 or BING packaging cells were transfected with retroviral plasmid vector

essentially as described by Pear et al. (1), except that 2 x HeBS buffer (25) was used instead of 2 x HBS. Murine packaging cell lines PE501 or PA317 were transfected by a standard calcium-phosphate precipitation protocol (25). Packaging cells were plated 24 hrs before transfection at a density of $2x10^6$ or $0.2x10^6$ per P60 plate for the human or mouse cells, respectively. The variations in the virus titers were achieved by using different amounts of vector DNA mixed with salmon sperm genomic DNA (carrier) up to the total DNA amount of 15 μg (higher amounts of vector DNA were used in some experiments, as indicated). Virus-containing media supernatants were collected 1 and 2 days after transfection.

In some experiments, the virus titers were determined by a colony-forming assay on NIH 3T3 cells. In this assay, 10^4 NIH 3T3 cells were plated in each well of a six-well plate. 24 hrs later, cells were infected with 2 ml of virus-containing media, prepared by serial dilutions of the corresponding supernatants from the transfected packaging cells. Cells were incubated with the virus in the presence of 4 μg/ml polybrene (Sigma) for 24 hrs. then with fresh media for another 24 hrs, and then with media containing 0.6 mg/ml G418 (active drug concentration) until the cell death was complete in control plates containing uninfected cells. G418-resistant colonies were stained with crystal violet and counted.

$1x10^5$ recipient HT1080 cells were plated per P60 24 hrs before infection. For infection, media on the plates were replaced on two consecutive days (unless indicated otherwise) with filtered virus-containing supernatants supplemented with 4 μg/ml polybrene. 24 hrs later. media were changed and the cells were allowed to grow in fresh media for 48 hrs more before splitting at $0.5x10^6$ cells per P100 (up to $2x10^6$ cells

per P100 were plated after infections with very low infection rate) and plating in the presence of 0.4 mg/ml (or other indicated effective concentrations) of G418. In each experiment, a control G418-containing plate was seeded with the same number of uninfected cells. G418-containing media were replaced every 3 days; the end of G418 selection was indicated by the time of complete death of uninfected cells. Unless indicated otherwise, the infected populations were maintained in the absence of G418 after the completion of the initial selection. In parallel with the selection of mass populations, the infection rate was determined by colony survival assays. For these assays, 500 cells were plated per P100 without G418 and several different cell numbers (from 10^3 to $2x10^6$ cells per P100, depending on the anticipated infection rate) were plated in the presence of G418. At the end of G418 selection, colonies were stained with crystal violet and counted.

Individual cell clones were derived in some experiments from G418-selected populations, by plating such populations at low density (50 cells per P100), in the absence of G418 and picking well-isolated colonies. In other experiments, subclones were isolated during the initial G418 selection, in parallel with the selection of mass populations, by picking individual colonies from G418-containing plates seeded at a lower density.

Luciferase assays. For luciferase assays, 0.5×10^6 aliquots of cells from different G418-selected populations or clones were collected in exponential phase of growth, suspended in 0.5 ml lysis buffer (Luciferase Assay System, Promega) and cleared lysates were frozen at -70°C until the assay. Luciferase activity was evaluated

using the Luciferase Assay System (Promega) according to the manufacturer's protocol, except that 5 μl of each sample and 50 μl of assay buffer were used for each assay. The luciferase activity was measured in a Beckman LS 5000TD scintillation counter. The results were normalized for the total protein amount in each sample, measured by a modified Bradford method using a Protein Assay Reagent (Bio-Rad). This assay was highly reproducible, as aliquoted frozen cell lysates, repeatedly measured at different times (up to 3 times over 6 months), showed ≤10% variability.

Southern hybridization. Genomic DNA was prepared using the Qiagen Blood and Cell Culture DNA kit (Qiagen). EcoRI or HindIII digested DNA was separated by electrophoresis in 0.8% agarose gel and transferred onto HybondN (Amersham) nylon membrane. Hybridization probes were prepared by restriction enzyme digestion of the LNCluc plasmid, followed by the isolation of the desired fragments by electrophoresis in 1% agarose gel and purification with a Qiaquick Gel Extraction kit (Qiagen). Probes were labeled with ^{32}P by random priming, using the Multiprime DNA Labeling System (Amersham). Hybridization and high-stringency washing were carried out as previously described (26).

Copy number of integrated proviruses was determined from Southern blots of EcoRI-digested DNA, by measuring hybridization signal intensities using Betascan (Betagen). The copy number for a given band was determined from its signal intensity relative to the signal intensity of the lanes with DNA from a cell line containing a single provirus (clone 1 or clone 2 derived from 0.2%-IIR population). As a control for variability of loading and transfer, the probe bound to the filters was allowed to decay

for three months and the filters were then rehybridized with an unrelated probe pMDR2 (27). Relative signal intensities for each band hybridizing with the retroviral probe were normalized for the intensity of the corresponding 3.1 kb pMDR2-specific band (28).

RESULTS

Relationship between the infection rate and the amount of vector DNA transfected into BOSC 23 packaging cells

Replication-deficient ecotropic retrovirus LNCluc (Fig. 1), carrying the firefly luciferase (*luc*) reporter gene under the control of the internal CMV promoter of the LNCX vector (20), was prepared in most experiments by transient transfection of the corresponding plasmid vector into ecotropic packaging cells BOSC 23, a derivative of 293 human renal cell line (1). As the recipient cell line, we have utilized subline pJet-2fTGH of human HT1080 fibrosarcoma cells (a gift of G.R. Stark), susceptible to ecotropic retrovirus and derived by transfecting 2fTGH subline of HT1080 (23) with the murine ecotropic receptor (24).

We were interested in determining the optimal amount of vector DNA for BOSC 23 transfection. For this purpose, recipient cells were infected on two consecutive days with virus-containing media supernatants collected 1 day and 2 days after transfection of BOSC 23 with different amounts of plasmid vector DNA. Infection rates were determined as the percentage of HT1080 cells surviving G418 selection, carried out at the minimal dose of drug that was required for complete killing of uninfected cells (0.4 mg/ml). Figure 2 shows the relationship between the amount of transfected DNA and

HT1080 infection rates in six experiments, carried out at different times using as vectors LNCX, LNCluc or LNCX carrying a short (188 bp) cDNA fragment of a mammalian gene. The infection rates reached a plateau at the amounts of transfected DNA that ranged from 0.1 to 5 μg per plate in different experiments. The infection rates at the plateau ranged from 31% to 78%. The lower infection rates were obtained in the experiments where BOSC 23 cells had been exposed to room temperature for a prolonged period of time: unless strictly maintained at 37°C, BOSC 23 is prone to lose the transgenes that express retroviral proteins (W.S. Pear, personal communication).

In several experiments, the virus yield was estimated on mouse NIH 3T3 cells by the formation of G418-resistant colonies (NIH 3T3 CFU assay). BOSC 23 cells, when rigorously kept at 37°C, consistently yielded a titer of $2x10^6$ to $5x10^6$ CFU/ml, even when the amount of transfected DNA was as low as 100 ng (data not shown). In three HT1080 cell infection experiments, where the apparent multiplicity of infection (m.o.i.) values (calculated from the NIH 3T3 CFU assays) were 30, 45 and 75, the respective HT1080 infection rates were 71%, 73%, and 41%. On the other hand, infection at a low m.o.i. (0.15) yielded HT1080 infection rate of 3%. Because of a lack of direct correlation between the m.o.i. and the infection rates (as also noted in ref. 29) only the HT1080 infection rates were determined for all of the experiments. These initial infection rates (IIR) were used as a characteristic of the G418-selected populations obtained from the corresponding infections. After the completion of the initial selection, the infected populations were maintained in the absence of G418 (except where indicated otherwise).

Relationship between IIR and reporter gene expression in G418-selected populations

We have measured luciferase activity in 36 populations of HT1080 pJet-2fTGH cells, that were independently infected with LNCluc at different IIR and selected with G418 (Fig. 3A,B). Even though G418 selection assured that each population consisted entirely of infected cells (as indicated by complete killing of uninfected cells in parallel plates), the luciferase activity varied widely among these populations. 9 populations expressed luciferase at very low levels (<300 cpm/pg protein) or not at all; all of these populations had IIR<1% (Fig. 3A). 9 of the other 10 populations with IIR≤5% showed luciferase activities ranging from 600 to 1200 cpm/pg, and one population had 1900 cpm/pg. There was no significant correlation between IIR and luciferase levels among the 19 populations with IIR≤5% (r^2=0.060) (Fig. 3A). In contrast, 15 of 17 populations with IIR≥6% expressed >2000 cpm/pg of luciferase, and the luciferase activity in these 17 populations was strongly correlated with IIR (r^2=0.837) (Fig. 3B).

Clonal variability in luciferase expression was analyzed in cell lines derived from infection experiments with high or low IIR values. Fig. 4A shows the luciferase levels in cell lines that were derived by subcloning (in the absence of G418) from G418-selected populations with a low (0.2%) and a high (31%) IIR. Clones derived from the high-IIR population showed highly variable luciferase expression levels, with the average level similar to the luciferase activity measured in the parental population. In contrast, 8 of 10 clones derived from the low-IIR population showed no luciferase expression, in accordance with a very low luciferase level in the parental population. Fig. 4B shows

the analysis of individual clones that were not subcloned from preselected populations but isolated as individual colonies after the initial G418 selection in two infection experiments with low IIR (0.005% and 0.7%) and one experiment with high IIR (22%). All three sets of clones showed a wide range of luciferase activity, but the luciferase levels in one half of the clones from the 22%-IIR infection were higher than in any of the clones from the low-IIR sets. Mass populations of infected cells were also derived by G418 selection (in parallel with the clones) from the infections with 0.7% and 22% IIR and maintained in tissue culture for the same time as the clones. While the luciferase activity of the 22%-IIR mass population closely matched the average for the individual clones selected in the same experiment, the luciferase level in the 0.7%-IIR mass population was lower than in any of the 13 clones isolated in parallel (Fig. 4B). The latter result suggests that the 0.7%-IIR population contained only a few clones with very low luciferase activity at the time of infection, but these clones had a growth advantage in the mass population.

Effects of G418 selection on the expression and stability of reporter gene expression

To determine whether G418 selection affected luciferase expression in the selected populations, we have measured the luciferase activity in 7 independently infected populations prior to the addition of G418. The expected luciferase activity in the infected cells was then estimated by dividing the measured luciferase levels by the IIR for the corresponding experiments, and this expected luciferase activity was compared with the actual activity measured in the same populations after G418

selection. Fig. 5 shows that the actual and expected activities were similar in the populations with 6% and 9% IIR, but the actual activity after G418 selection gradually decreased (relative to the expected level) in populations with lower IIR. Thus, G418 selection was detrimental to luciferase expression in populations with low IIR, but had no major effect on higher-IIR populations.

We have also analyzed the stability of luciferase expression in 11 G418-selected populations with IIR ranging from 0.1% to 33%. Cells were maintained in the presence or in the absence of G418 up to 76 days after completion of the initial G418 selection (defined as the time when no viable cells remained in uninfected control plates), and luciferase levels were measured on days 12, 19, 36 and 76 (Fig. 6). When maintained in the absence of G418, 8 populations with 1%-33% IIR showed no significant change in luciferase expression between days 12 and 36, though by day 76 these populations showed a moderate (11%-54%) decrease relative to day 12. In contrast, three populations with very low IIR (0.1%, 0.2% and 0.6%) showed continuous loss of luciferase expression in the absence of G418, with 83%-97% of activity lost by day 76. Since the latter populations included a high proportion of cells with no luciferase expression (see Fig. 4A for clonal analysis of the 0.2%-IIR population), this loss can be explained by random "drift" that occured during the passaging of cells, rather than intrinsic instability of luciferase expression in the infected cells. In agreement with this interpretation, two luciferase-expressing clones isolated from the 0.2%-IIR population showed no decrease in luciferase activity over a period of 45 days after subcloning (data not shown).

Parallel cell cultures, maintained in the presence of G418, showed much more rapid loss of luciferase expression in most of the populations, except for three populations with the highest IIR (22%, 31% and 33%). In particular, three populations with the lowest IIR values lost their luciferase activity completely or nearly completely by day 36, when grown in the presence of G418 (Fig. 6). This result further indicates that exposure of low-IIR populations to G418 provides a selective advantage for cells with very low luciferase activity.

Lack of reporter gene expression in low-IIR populations is associated with proviral DNA rearrangements

To determine if differences in luciferase expression reflected changes in the copy number or structural integrity of integrated proviruses, Southern blot analysis was carried out on genomic DNA from different infected populations or clones. Two types of assays were used (Fig. 1). In the first assay, genomic DNA was digested with EcoRI, which cuts twice within LNCluc, excising an internal 2.6 kb fragment that contains the coding sequence of *neo*, the internal CMV promoter and the 5' part of *luc*. A probe corresponding to this fragment would hybridize with the same 2.6 kb band for each integrated provirus (unless proviral DNA is rearranged), with the band intensity proportional to the provirus copy number. In the second assay, genomic DNA was digested with HindIII, which cuts once in the LNCluc provirus, between the CMV promoter and the luciferase gene. A probe corresponding to the 2.0 kb EcoRI-HindIII fragment of LNCluc would hybridize with different bands for each integrated provirus, since the size of each band would be determined by the location of a HindIII site in the

flanking cellular sequences (Fig. 1). Thus, the number of different hybridizing fragments in the HindIII digest would correspond, in the first approximation, to the number of different integration events (as limited by the resolution of Southern blots).

We have applied this analysis to a low (0.2%) IIR population and cellular clones derived from it. EcoRI analysis of 10 clones from this population (Fig. 7A, lanes 1-10) revealed that only one of them, clone 2 showed the expected single band of 2.6 kb size (lane 2). The other clones showed instead a major band of approximately 3.5 kb size, suggesting a common rearrangement in the provirus carried by these clones. This conclusion was confirmed by HindIII analysis (Fig. 7B, lanes 2-7), where each of the tested clones showed one 11 kb band, indicative of a single provirus integration. This band was seen in all the clones other than clone 2 which instead contained a 8.4 kb band (lane 3 in Fig. 7B), indicating that all the clones with the rearranged provirus had a common origin. HindIII and EcoRI analysis of the DNA from the parental 0.2%-IIR cell population shows that the bands corresponding to the rearranged provirus were predominant in this population prior to cloning (lane 1 in Fig. 7B and lane 2 in Fig. 7C). The 0.2%-IIR population, grown for 76 days after the removal of G418 still contains the unrearranged 2.6 kb band as a minor component (lane 2 in Fig. 7C), but this band is undetectable in cells maintained for the same time in the presence of G418 (lane 3 in Fig. 7C).

The luciferase activity of the clones derived from the 0.2%-IIR population is illustrated in Fig. 4A. Only clone 2, which shows no provirus rearrangement, possessed significant luciferase activity. The other clones were completely negative, except for clone 5 which showed very low luciferase activity. EcoRI analysis of this

clone (lane 5 in Fig. 7A) shows that clone 5 contains a weak band corresponding to the unrearranged 2.6 kb fragment, in addition to the major 3.5 kb rearranged band. This clone therefore appears to be not a pure cell line but rather an unequal mixture of two cellular clones. PCR analysis of genomic DNA from the rearranged and unrearranged clones derived from the 0.2%-IIR population showed that the rearrangement, while leaving the *neo* gene intact, involves a complete or partial loss of the CMV promoter (data not shown), thus accounting for the lack of luciferase expression from the rearranged provirus. Thus, G418 selection of the 0.2%-IIR population has enriched for a clone containing a specific DNA rearrangement that abolishes luciferase expression from the integrated provirus.

In contrast to the 0.2%-IIR population, another low (0.7%) IIR population was found by EcoRI analysis to contain primarily unrearranged provirus, though a minor subpopulation with a specific rearrangement was also detectable in these cells (lane 6 in Fig. 7C); the 0.7%-IIR population also showed higher luciferase expression than the 0.2%-IIR population. We have also analyzed a very low (0.02%) IIR population, which was obtained after infection of the original HT1080 cell line (without ecotropic receptor) with amphotropic LNCluc virus, derived by transient transfection of BING packaging cells (21). As shown in Fig. 7D (lane 2), this population, like the 0.2%-IIR population, contained a single predominant rearranged band (3.4 kb size), which was more intense than the unrearranged 2.6 kb band present in the same population. Lanes 3-6 in Fig. 7D correspond to 4 clones that were selected with G418 as individual colonies after the same 0.02%-IIR infection. In contrast to the population, these clones showed only the unrearranged 2.6 kb band. Two of these clones expressed detectable luciferase levels.

while 2 other clones did not express luciferase at all despite the apparently intact provirus. Thus, loss of expression from an internal promoter in our system does not necessarily involve a gross DNA rearrangement.

Reporter gene expression in high-IIR populations correlates with the copy number of unrearranged proviruses

EcoRI analysis of 10 clones derived from a high (31%) IIR population (lanes 11-20 in Fig. 7A) showed that all of these clones contained the unrearranged 2.6 kb band, but three of the clones (lanes 11, 13, 16) contained additional rearranged bands of different sizes. The uncloned 31%-IIR population also showed the predominance of the unrearranged band (lane 7 in Fig. 7C), though growth of this population for 76 days in the presence of G418 led to the appearance of prominent secondary bands corresponding to rearranged proviruses (lane 8 in Fig. 7C). Furthermore, quantitative analysis of signal intensity of the 2.6 kb band (using a probe corresponding to a fragment of the MDR2 gene as a normalization control) indicated that the unrearranged provirus was present at an average of 4.4 copies per cell in the 31%-IIR population and at different copy numbers (1-9 copies per cell) in individual clones. These conclusions were confirmed by HindIII analysis (lanes 8-14 in Fig. 7B), which showed a continuous smear (indicative of a very large number of provirus integration sites) in the uncloned 31%-IIR population (lane 14), and multiple discrete bands in most of the tested clones from this population (lanes 9-13).

EcoRI analysis of other populations with high IIR values showed that the presence of multiple copies of unrearranged provirus is common in such populations.

Thus, a 16%-IIR population (lane 11 in Fig. 7C) contained an average of 2.5 copies of unrearranged provirus per cell, with no detectable rearrangements. As with the 31%-IIR population, prominent rearranged bands became apparent in this population after continuous cultivation in the presence of G418 (lane 12 in Fig. 7C). Similarly, a 22%-IIR population (lane 13 in Fig. 7C) contained an average of 1.9 provirus copies per cell, without major rearrangements. Surprisingly, two populations with IIR as low as 6% contained an average of 2.4 or 2.6 provirus copies per cell, indicating that multicopy integration does not require high infection efficiency. One of these 6%-IIR populations (obtained after amphotropic virus infection of the original HT1080 cells) is shown in lane 7 of Fig. 7D (2.6 copies per cell), while 4 clones isolated in parallel with this population (lanes 8-11 in Fig. 7D) contained 1.5-5.2 copies per cell. As shown in Fig. 8, there is a general (though imperfect) correlation between IIR and the copy number of integrated unrearranged proviruses in 9 tested populations (r^2=0.654).

Fig. 9 shows a comparison between the copy number of unrearranged proviruses and the luciferase activity in 5 populations and 9 clones of HT1080 pJet-2fTGH cells that contained more than one unrearranged provirus per cell. Luciferase expression was strongly correlated with the copy number of unrearranged proviruses (r^2=0.879), indicating that the provirus copy number is the primary determinant of luciferase expression in high-IIR populations. It should be noted, however, that LNCluc-infected clones of the original HT1080 cell line expressed 2-3 times less luciferase per provirus copy than did the cells of the pJet-2fTGH subline (data not shown), indicating the importance of cell-specific factors in gene expression from retroviral vectors.

Effects of increased stringency of G418 selection on luciferase expression and provirus rearrangements

The results described above indicate that G418 selection of infected populations under standard conditions (0.4 mg/ml) is detrimental to the expression from the internal CMV promoter in low-IIR populations. In populations with IIR≥6%, however, G418 selection had no major effect on luciferase expression, though prolonged exposure of such populations to G418 caused a noticeable decrease in luciferase expression and emergence of cell subpopulations with rearranged proviruses.

To determine how luciferase expression would be affected by increasing the concentration of G418 used in the original selection, LNCluc virus, derived by BOSC 23 transfection, was used to infect recipient cells either once (1 day or 2 days post-transfection) or twice (on days 1 and 2). The infected cells were then selected at a range of different G418 concentrations, from the standard 0.4 mg/ml up to 5 mg/ml G418. The IIR values, as determined at 0.4 mg/ml G418, were 9% for cells infected on day 1, 4% for cells infected on day 2, and 6% for cells infected on day 1 and day 2. As expected, the survival fraction in all three infected populations decreased with increasing concentrations of G418 (data not shown).

The luciferase activity of populations selected at different G418 concentrations showed an overall decrease with increasing G418 concentrations, but the extent of the decrease varied among the three populations (Fig. 10A). While the population infected on day 2 showed a complete loss of luciferase expression at the highest G418 concentration (5 mg/ml), the luciferase levels in the populations infected on day 1 and

selected with 2.5-5 mg/ml G418 stabilized at the plateau corresponding to approximately 20% of the expression observed at 0.4 mg/ml. The decrease was much less pronounced in the doubly-infected population, where luciferase expression at G418 concentrations from 1.5 to 5 mg/ml showed a plateau corresponding to approximately 50% of the level obtained at 0.4 mg/ml.

An interesting picture emerged from the analysis of luciferase expression in cell lines subcloned (in the absence of G418) from the populations that were infected on day 1 or on day 1 plus day 2 and selected at 0.4, 2.5 or 5 mg/ml G418 (Fig. 10B,C). Most of the clones isolated from both populations selected at 0.4 mg/ml showed intermediate levels of luciferase expression. In contrast, almost all the clones from the singly-infected population were luciferase-negative when selected at 2.5 or 5 mg/ml G418 (Fig. 10B). Increasing G418 concentration, however, had a different effect on doubly-infected cells (Fig. 10C). At 2.5 mg/ml, 5 of 8 clones in this group showed intermediate luciferase activity, but 2 clones showed high levels of luciferase and one clone was negative. At 5 mg/ml, 2 of 7 clones became luciferase-negative, 2 clones showed low luciferase activity, and 3 clones expressed a high level of luciferase. This analysis suggests that increasing concentrations of G418 may select for both luciferase-negative clones and for clones expressing higher than average levels of luciferase.

EcoRI Southern hybridization analysis was carried out on the populations infected on day 1 and on day 1 plus day 2 and selected at 0.4, 2.5 or 5 mg/ml G418 (lanes 14-19 in Fig. 7D). Both of the populations selected in 0.4 mg/ml G418 contained primarily the unrearranged (2.6 kb) band, with the intensity corresponding to 2.4 copies

per cell in both cases (lanes 14,15). In contrast, populations selected at 2.5 or 5 mg/ml G418 developed prominent rearranged bands (lanes 16-19). The appearance of the rearranged bands and, in some cases, a decrease in the intensity of the apparently unrearranged band, paralleled the observed decrease in the luciferase activity of the corresponding populations.

DISCUSSION

In the present study, we have analyzed the effect of differences in the infection rate and G418 selection conditions on the expression of a reporter gene (luciferase) from an internal promoter of a double-gene retroviral vector, LNCX, transduced into human HT1080 fibrosarcoma cells. Our study was deliberately limited to a single recipient cell/vector combination, since we wanted to use a clearly defined experimental system to identify the factors responsible for experimental variability in the expression of retrovirally transduced genes. Nevertheless, the types of problems that were identified in our study (instability of gene expression from retroviral vectors, poor expression of the nonselected gene, proviral DNA rearrangements) have been commonly observed with many different recipient cell types and with other classes of double-gene retroviral vectors. We believe therefore that the general correlations observed in this work will be applicable in principle to other cell/vector systems, and should be considered in designing functional assays for genes or genetic elements transduced by retroviral vectors.

The first non-obvious result of our study is that different retrovirally infected cell populations, selected with G418 and therefore consisting entirely of transduced cells, showed widely varying levels of luciferase expression, and in some cases did not express luciferase at all. Also unexpectedly, these differences turned out to correlate with the IIR of the tested populations. In particular, all the populations with very low or no luciferase expression arose from the infections with IIR <1%. Furthermore, populations with IIR≤5% showed lower luciferase expression than the populations with IIR≥6%, and the levels of expression in the latter group were significantly correlated with their IIR values. Thus, IIR determination can be used to identify populations that may fail to express the nonselected gene, and to gauge the likely relative levels of gene expression among independently infected populations. We would expect, however, that the quantitative parameters of the correlation between IIR and gene expression would be different for different cell/vector systems, as we have observed even for two different sublines of HT1080 cells infected with the same retroviral vector.

G418-selected populations with low IIR, as well as clones derived from such populations, showed single-copy provirus integration, with a high incidence of proviral DNA rearrangements. Provirus rearrangements in double gene vectors have been previously observed by other investigators (7, 12). These rearrangements affect the transcription unit driven by the internal promoter and therefore abolish reporter gene expression. On the other hand, some of the clones that did not express luciferase showed apparently intact proviral DNA, suggesting that the internal promoter in these clones was inactivated without a major rearrangement, as previously observed in other cell/vector systems (5, 7). Clonal analysis of low-IIR populations demonstrated that

they generally consist of two types of cells: those that do not express luciferase at all, and those that contain a single copy of an unrearranged provirus and express luciferase at an intermediate level. The levels of luciferase expression, which vary widely among low-IIR populations appear to be determined primarily by the ratios of these two cell types within each population. As will be discussed elsewhere in this section, the loss of luciferase expression in low-IIR populations is a consequence of G418 selection.

Analysis of genomic DNA from the high-IIR populations demonstrated that all the tested populations with IIR≥6% contained between 2.0 and 4.4 copies of integrated unrearranged provirus per cell. The provirus copy number in different populations showed some correlation with IIR. More importantly, the levels of luciferase expression among the high-IIR populations and the corresponding clones were strongly correlated with the copy number of unrearranged proviruses. This suggests that the copy number is the primary determinant of gene expression in these populations, or in single clones containing multiple integrated proviruses. On the other hand, such factors as the provirus integration site are likely to be responsible for the variability in gene expression among cell clones containing a single copy of unrearranged provirus.

Multicopy integration of retroviral vectors has been previously demonstrated not only at the level of individual clones, but also in the total infected populations. In such populations, however, the observed infection rates were 80% (7) or 100% (14). In contrast, we have detected multicopy integration at infection rates as low as 6% and at m.o.i. as low as 0.5 (as calculated for a 6%-IIR population of HT1080 cells that were infected with amphotropic virus and acquired an average of 2.6 proviruses per cell).

Multicopy integration in such populations most probably is not a consequence of preferential G418 selection of cells with more than one provirus, since in such a case one could expect the luciferase levels to be increased in G418-selected relative to unselected populations. However, luciferase levels measured before and after G418 selection in two independent populations that acquired 2.4 provirus copies per cell were in close agreement. Multicopy integration occurring at infection rates that are much lower than 100% suggests that the recipient cells contain a subpopulation which is preferentially susceptible to retroviral infection, and which is likely to acquire two or more proviruses while the bulk of the population remains uninfected. The remaining cells, however, are not entirely refractory to retroviral infection, since up to 78% of the recipient cells could be infected at higher titers of LNCluc. Retroviral infectability has been related to the distribution and interactions of viral receptors on the cell surface (30, 31), metabolic factors affecting reverse transcription (32, 33) or provirus integration (34), the ability of cells to divide (35), and phase of the cell cycle (36).

G418 selection, however, appears to play a critical role in enriching for cells that have lost the internal promoter function. Thus, the luciferase levels measured after G418 selection of cell populations with IIR<2-3% were much lower than the levels calculated from the luciferase measurements conducted before G418 selection. While G418 selection under standard conditions showed no significant effect on cell populations with higher IIR, prolonged growth of such populations in the presence of G418 led to a partial loss of luciferase expression and an emergence of rearranged proviral sequences, in agreement with the previous observation of Olsen et al. (12). An increase in the G418 concentration used for the initial selection of populations with

higher IIR had a dual effect. Higher G418 concentrations increased the proportion of cells that have lost luciferase expression, which was accompanied by an increase in proviral DNA rearrangements. This negative effect of high G418 concentrations appears to be partially offset, however, by concurrent selection of a subpopulation of cells with increased luciferase expression, presumably reflecting a higher copy number of unrearranged proviruses in such cells.

The observed differential effects of G418 selection on luciferase expression in populations with different IIR can be explained by considering the nature of G418 selection. The drug G418 (a translation inhibitor) induces either complete or partial growth inhibition in mammalian cells, depending on the intracellular drug concentration and the intrinsic cellular sensitivity to this drug. The bacterial *neo* gene encodes neomycin phosphotransferase, an enzyme that metabolically inactivates G418, with the extent of inactivation dependent on the drug concentration and on the amount of enzyme produced by *neo*-expressing cells. Under our empirically determined selection conditions (0.4 mg/ml G418), there is no survival of HT1080 cells that do not contain *neo*, but cells carrying at least one copy of a *neo*-expressing provirus are able to grow. However, cells that express an elevated level of neomycin phosphotransferase (either through the presence of more than one copy of *neo* or through some changes increasing the expression of the single *neo* gene) are apt to have a growth advantage in the presence of the drug, as they inactivate G418 more efficiently (9). Since two adjacent promoters present in the same vector can interfere with each other (5), a rare event silencing the internal CMV promoter (through proviral DNA rearrangement or some other mechanism) is likely to increase the expression of the *neo* gene and

therefore provide cells with a growth advantage in the presence of G418. As a result, such cells become enriched after G418 selection of low-IIR populations (where multicopy integration is very rare). In contrast to the low-IIR populations, most of the cells infected at high IIR contain two or more copies of the provirus, which provides them with an adequate level of *neo* expression for optimal growth. As a result, cells with inactivation of the internal promoter do not show significant enrichment in high-IIR populations under standard selection conditions. Nevertheless, such cells gain a selective advantage with an increase in the stringency or the duration of G418 selection, as has been observed in our study.

It is conceivable that some of the problems that we have characterized could be diminished or obviated through changes in the vector design. An attractive alternative to the selectable drug-resistance genes is provided by fluorescent markers, such as the green fluorescent protein (37) or physiologically neutral cell surface antigens detectable by immunofluorescence (7). Retrovirally transduced cells expressing such markers can be isolated by fluorescence-activated cell sorting shortly after infection, and no continuous selection would be required for these markers. Another general change would be in the relative position of the two genes. Some investigators have reported improved expression of the nonselected gene when such a gene is expressed from the LTR and the selected gene from an internal promoter (7, 8, 13), though others have reported the problems of poor expression, rearrangement or instability with such vectors as well (5, 7). In our hands, a vector of this type, LXSN (20), does not appear to provide an improvement over LNCX in several tested cell lines, but we have not analyzed the LXSN vector systematically. Finally, vectors expressing both genes from a

single bicistronic message, using an IRES for translation initiation of the second gene, would in principle avoid the problem of "promoter competition" altogether, and they have in fact been reported to provide improved coexpression of the two genes relative to two-promoter vectors (3, 38). Due to the relative novelty of such vectors, however, it is still unclear how they compare to conventional vectors in the expression stability or viral titers. At any rate, double-gene vectors with the gene of interest transcribed from an internal promoter, still remain the primary choice in the cases where the LTR promoter does not provide adequate expression in the target cell type, or when the internal promoter is chosen for tissue-specific or inducible expression.

Based on the results of the present study, some general recommendations can be made for the functional analysis of genes transduced with double-gene retroviral vectors. First of all, one should keep in mind the possibility that some mass populations transduced with such a vector and selected for the function of one of the genes, may express the nonselected gene poorly or not at all, even if the same cell/vector combination has produced adequate expression in previous experiments. To avoid this problem, an ideal solution in theory would be to carry out the infection at a very high virus titer or in multiple rounds, so as to infect all the recipient cells and avoid selection altogether. Unfortunately, this solution is not feasible for most of the recipient cell types, in particular for cells that grow in suspension. Given the necessity of selection, it is important to maximize the virus titer so as to achieve an infection rate high enough to produce an average of more than one copy of integrated provirus per infected cell (as can be determined by DNA hybridization). Our results indicate that multicopy integration (at least in HT1080 cells) can be achieved with either ecotropic or

amphotropic retrovirus even when the infection rate is relatively low. Furthermore, it would be helpful to minimize the stringency and the duration of selection for the selectable gene of the retroviral vector. When a cytotoxic drug (such as G418) is used for selection, the lowest dose and time of exposure sufficient to kill the uninfected cells should be determined for each recipient cell line, and the stringency of selection should not be increased. Finally, we would recommend assaying the RNA (or protein) expression of the nonselected gene in each transduced population, to assure consistent interpretation of different infection experiments.

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LITERATURE CITED

1. Pear W.S., Nolan G.P., Scott M.L. and Baltimore D. (1993). *Proc. Natl. Acad. Sci. U.S.A.* **90:** 8392-8396.
2. Soneoka Y., Cannon P.M., Ramsdale E.E., Griffiths J.C., Romano G., Kingsman S.M. and Kingsman A.J. (1995). *Nucleic Acids Res.* **23:** 628-633.
3. Ghattas I.R., Sanes J.R. and Majors J.E. (1991). *Mol. Cell Biol.* **11:** 5848-5859.
4. Morgan A., Couture L., Elroy-Stein O., Ragheb J., Moss B. and Anderson W.F. (1992). *Nucleic Acids Res.* **20:** 1293-1299.
5. Emerman M. and Temin H.M. (1984). *Cell* **39:** 459-467
6. Chang S.M., Wager-Smith K., Tsao T.Y., Henkel-Tigges J., Vaishnav S. and Caskey C.T. (1987). *Mol. Cell Biol.* **7:** 854-863.
7. Xu L., Yee J.K., Wolff J.A. and Friedmann T. (1989). *Virology* **171:** 331-341.
8. Bowtell D.D., Cory S., Johnson G.R.and Gonda T.J. (1988). *J. Virol.* **62:** 2464-2473.
9. Paludan K., Duch M., Jorgensen P., Kjeldgaard N.O.and Pedersen F.S. (1989). *Gene* **85:** 421-426.
10. Hoeben R.C., Migchielsen A.A., van der Jagt R.C., van Ormondt H. and van der Eb A.J. (1991). *J. Virol.* **65:** 904-9012.
11. Moolten F.S., Wells J.M. and Mroz P.J. (1992). *Cancer Letters* **64:** 257-263
12. Olsen J.C., Johnson L.G.,Wong-Sun M.L., Moore K.L., Swanstrom R. and Boucher R.C. (1993).*Nucleic Acids Res.* **21:** 663-669.

13. McLachlin J.R., Mittereder N., Daucher M.B., Kadan M.and Eglitis M.A. (1993). *Virology* **195:** 1-5.

14. Correll P.H., Cololla S. and Karlsson S. (1994). *Blood* **84**: 1812-1822.

15. Molina R.M., Cheboune Y., Verdier G. and Legras C. (1995). *C. R. Acad. Sci. III* **318:** 541-551

16. Sadelain M., Wang C.H., Antoniou M., Grosveld F. and Mulligan R.C. (1995). *Proc. Natl. Acad. Sci. U.S.A.* **92:** 6728-6732.

17. Gudkov A.V., Kazarov A.R., Thimmapaya R., Axenovich S.A., Mazo I.A. and Roninson I.B. (1994). *Proc. Natl. Acad. Sci. U.S.A.* **91:** 3744-3748.

18. Gudkov A.V., Zelnick C.R., Kazarov A.R., Thimmapaya R., Suttle D.P., Beck W.T. and Roninson I.B. (1993). *Proc. Natl. Acad. Sci. U.S.A.* **90:** 2131-3235.

19. Roninson I.B., Gudkov A.V., Holzmayer T.A., Kirschling D.J., Kazarov A.R., Zelnick C.R., Mazo I.A., Axenovich S. and Thimmapaya R. (1995). *Cancer Res.* **55:** 4023-4028.

20. Miller D.A and Rosman G.J. (1989). *Biotechniques* **7:** 980-986.

21. Pear W.S., Scott M.L., and Nolan G.P. (1996). In: *Methods in Molecular Biology, Vol. X: Methods in Gene Therapy,* P. Robbins, ed., Humana Press, Totowa, NJ, in press.

22. Bender M.A., Palmer T.D., Gelinas R.E. and Miller A.D.(1987). *J. Virol.* **61**:1639-1646.

23. Pellegrini S., John J., Shearer M., Kerr I.M. and Stark G.R. (1989). *Mol. Cell. Biol.* **9**: 4605-4612.

24. Albritton L.M., Tseng L., Scadden D., and Cunningham J.M. (1989). *Cell* **57:** 659-666.

25. Kingston R.E. (1987). In: *Current Protocols in Molecular Biology*, eds. F.M. Ausubel, R. Brent, R.E. Kingston and D.D. Moore, Current Protocols, New York, pp. 9.1.1-9.1.4.

26. Schott B., Bennis S., Pourquier P., Ries C., Londos-Gagliardi D. and Robert J. (1993). *Int. J. Cancer* **55:** 115-121.

27. Roninson I.B., Chin J.E., Choi K.. Gros P., Housman D.E., Fojo A., Shen D., Gottesman M.M., and Pastan I. (1986). *Proc. Natl. Acad. Sci. USA* **83:** 4538-4542.

28. Choi, K., Hake, L.E., Bowcock. A.M., Roninson, I.B., and Cavalli-Sforza, L.L. (1987). *Nucleic Acids Res.* **15**: 6305.

29. Morgan J.R., LeDoux, J.M., Snow R.G., Tompkins R.G., and Yarmush, M.L. (1995). *J. Virol.* **69**: 6994-7000.

30. Wang H., Paul R., Burgeson R.E., Keene D.R. and Kabat D. (1991). *J. Virol.* **65**: 6468-6477.

31. Woodard M.H., Dunn W.A., Laine R.O., Malandro M., McMahon R., Simell O., Block E.R. and Kilberg M. (1994). *Am. J. Physiol.* **266:** E817-E824.

32. Goulaouic H., Subra F., Mouscadet J.F., Carteau S. and Auclair C. (1994). *Virology* **200**: 87-97.

33. Zhang H., Duan L.X., Dornadula G.and Pomerantz R.J. (1995). *J. Virol.* **69**: 3929-3932.

34. Bouille P., Subra F., Goulaouic H., Carteau S. and Auclair C. (1995) *Cancer Res.* **55:** 3211-3217.

35. Miller D.G., Adama M.A., and Miller A.D. (1990). *Mol. Cell. Biol.* **10:** 4239-4242.

36. Springett G.M., Moen R.C., Anderson S., Blaese R.M. and Anderson W.F. (1989). *J. Virol.* **63**: 3865-3869.

37. Chalfie M., Tu Y., Euskirchen G., Ward W. W. and Prasher D. C. (1994). *Science* **263**: 802-805

38. Metz M.Z., Matsumoto L., Winters K.A., Doroshow J.H. and Kane S.E.(1996) *Virology* **217**: 230-241.

FIGURE LEGENDS

FIG. 1. Map of integrated LNCluc provirus and probes used for Southern hybridization analysis. The arrows show the positions of transcription initiation for the two promoters. Dashed lines indicate the flanking cellular sequences. The 2.0 kb probe was used for hybridization with HindIII digests of genomic DNA, where the size of its hybridizing fragments depends on the position of a HindIII site in the upstream cellular flanking sequences (placed at an arbitrary location in the figure). The 2.6 kb probe was used for hybridization with EcoRI-digested DNA, where, in the absence of proviral rearrangement, it hybridizes to a band of the same size.

FIG. 2. Relationship between the infection rate and the amount of vector DNA transfected into BOSC 23 packaging cells. The vectors used were LNCluc (squares), LNCX (circles), and LNCX carrying a short cDNA insert (diamonds). The infection rates were determined as the percentage of HT1080 cells surviving G418 selection.

FIG. 3. Relationship between IIR and luciferase activity in 36 mass populations of HT1080 pJet-2fTGH cells independently infected with LNCluc, with IIR≤5% (A) or IIR≥6% (B). Populations obtained after infection utilizing BOSC 23 packaging cells are indicated with open squares, and populations derived through PE501 or PA317 packaging lines are indicated with closed squares or closed circles, respectively. Luciferase activity was measured on cells recovered in an exponential phase of growth 3-6 days after the completion of G418 selection.

FIG. 4. Luciferase activity in clonal cell lines derived from different mass populations of infected cells. Each set is designated by the IIR values (%) of the corresponding populations. Squares indicate the luciferase activity of individual clones, and horizontal lines show the activity of the corresponding populations. Clones in (A) were derived by subcloning from the corresponding G418-selected populations; clones in (B) were isolated as individual colonies in parallel with the corresponding mass populations. Clones 2 and 5 from the 0.2%-IIR population are labeled in panel A.

FIG. 5. Luciferase activity in HT1080 pJet-2fTGH cell populations before and after G418 selection. For each population (with the indicated IIR), the estimated luciferase activity of infected cells before G418 selection is shown on the left (light bars), and the actual luciferase activity measured 3-6 days after G418 selection is shown on the right (dark bars). The estimated activity before selection was determined by measuring the luciferase activity 3 days after the infection, prior to the addition of G418, and dividing this value by the IIR determined for the same infection.

FIG. 6. Stability of luciferase expression in G418 selected populations of HT1080 pJet-2fTGH cells. The IIR values for the corresponding populations are indicated in boldface. The Y axis represents luciferase activity (in cpm/pg protein) for the corresponding cell populations, measured on days 12, 19, 36 and 76 after the completion of G418 selection, as indicated by numbers on top of the bars. Light and

dark bars correspond to cell populations maintained in the absence or in the presence of G418, respectively.

FIG. 7. Southern hybridization analysis of HT1080 cell clones and populations infected with LNCluc retrovirus. Except where indicated, infected cells were derived from HT1080 pJet-2fTGH cell line. Genomic DNA (10 mg) was digested with EcoRI (panels A, C and D) or HindIII (Panel B). EcoRI digests were hybridized with a 2.6 kb probe from LNCluc (Fig. 1) (above) and subsequently with a control pMDR2 probe (below). HindIII digests were hybridized with a 2.0 kb probe from LNCluc (Fig. 1). The sizes of the bands mentioned in the text are indicated. Individual lanes contain DNA from the following sources.

A. 1-10, clones 1-10 (respectively) from 0.2%-IIR population; 11-20, clones 1-10 (respectively) from 31%-IIR population.

B. 1, 0.2%-IIR population; 2-7, clones 1,2,4,5,9 and 10 (respectively) from 0.2%-IIR population; 8-13, clones 1,2,3,6,7,8 (respectively) from 31%-IIR population; 14, 31%-IIR population.

C. 1, uninfected HT1080 cells; 2, 0.2%-IIR population; 3, 0.2%-IIR population, cultured for 76 days in the presence of G418; 4 and 5, clones 1 and 2 (respectively) from 0.2%-IIR population; 6, 0.7%-IIR population; 7, 31%-IIR population; 8, 31%-IIR population, cultured for 76 days in the presence of G418; 9 and 10, clones 1 and 8 (respectively) from 31%-IIR population; 11, 16%-IIR population; 12, 16%-IIR population, cultured for 76 days in the presence of G418; 13, 22%-IIR population.

D. 1, uninfected HT1080 cells (original line); 2, 0.02%-IIR population (derived from original HT1080 cell line); 3-6, clones 1,2,3,4 (respectively) from 0.02%-IIR population; 7, 6%-IIR population (infection of original HT1080 cell line); 8-11, clones 1,2,3,4 (respectively) from 6%-IIR population; 12, clone 1 from 0.2%-IIR population; 13, clone 8 from 31%-IIR population; 14, cell population infected on day 1 (9% IIR) and selected with 0.4 mg/ml G418; 15, cell population infected on day 1 plus day 2 (6% IIR) and selected with 0.4 mg/ml G418; 16, cell population infected on day 1 and selected with 2.5 mg/ml G418; 17, cell population infected on day 1 plus day 2 and selected with 2.5 mg/ml G418; 18, cell population infected on day 1 and selected with 5 mg/ml G418; 19, cell population infected on day 1 plus day 2 and selected with 5 mg/ml G418;

FIG. 8. Relationship between IIR and the average unrearranged provirus copy number in LNCluc-infected mass populations. Populations derived from HT1080 pJet-2fTGH subline are indicated with squares and populations from the original HT1080 line are indicated with circles.

FIG. 9. Relationship between unrearranged provirus copy number and luciferase expression in different clones and populations of LNCluc-infected HT1080 pJet-2fTGH cells. Cell populations are indicated with open squares and clones with closed squares.

FIG. 10. Luciferase expression in LNCluc-infected HT1080 pJet-2fTGH cells, selected at different G418 concentrations.

A. Relationship between selective concentration of G418 (mg/ml) and luciferase activity in cell populations infected on day 1 (open squares), day 2 (half-open squares) and on day 1 plus day 2 (closed squares) after BOSC 23 transfection. Luciferase activity was determined 5 days after the completion of G418 selection.

B and C. Luciferase expression in clones isolated from the populations infected on day 1 (B) or on day 1 plus day 2 (C) and selected with the indicated G418 concentrations.

Figure 1

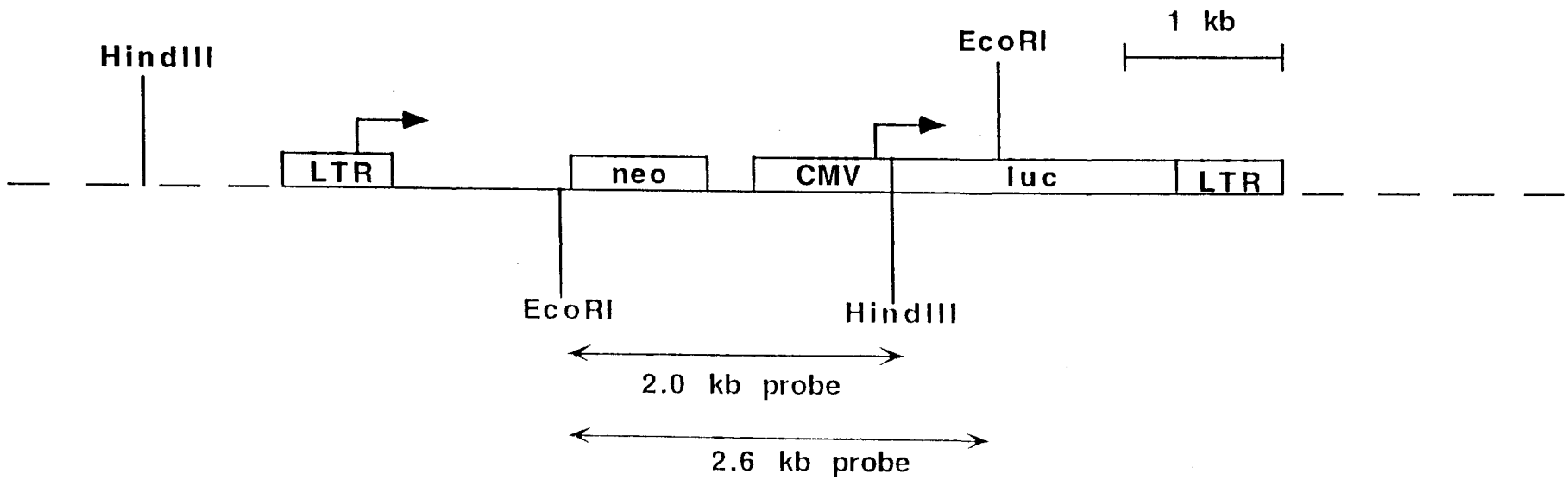
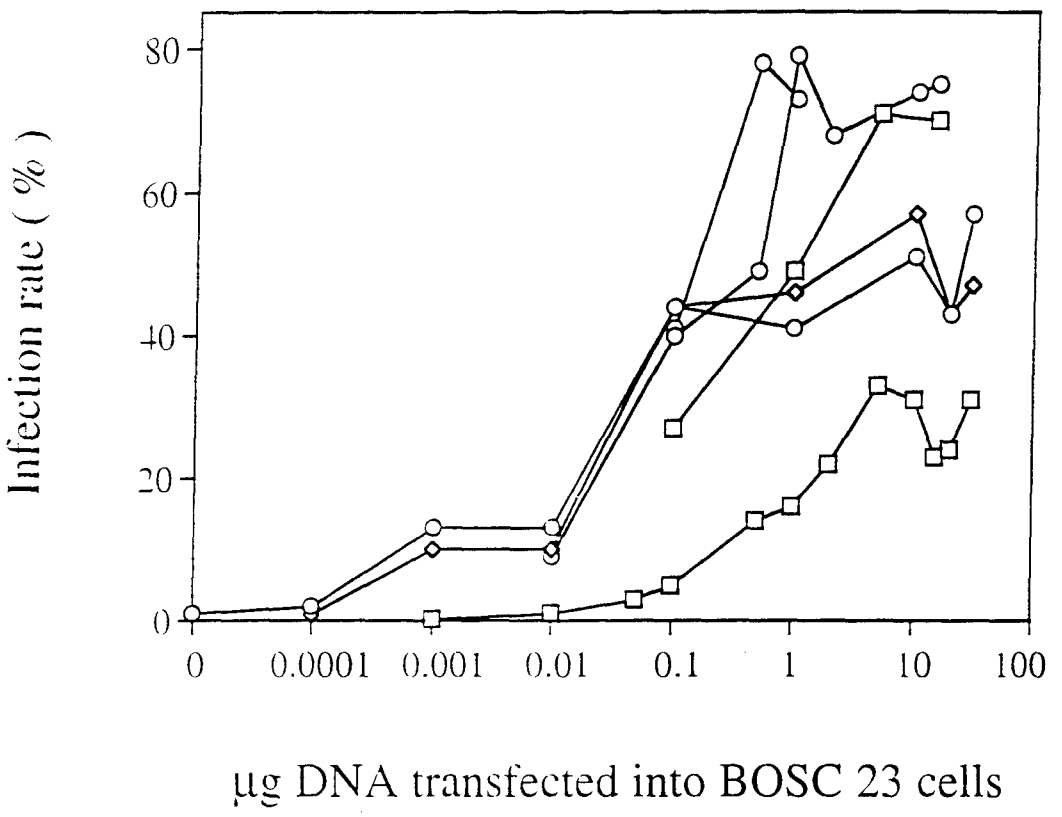

HindIII
EcoRI
1 kb
LTR
neo
CMV
luc
LTR
EcoRI
HindIII
2.0 kb probe
2.6 kb probe

Figure 2


Infection rate (%)
80
60
40
20
0
0
0.0001
0.001
0.01
0.1
1
10
100
µg DNA transfected into BOSC 23 cells

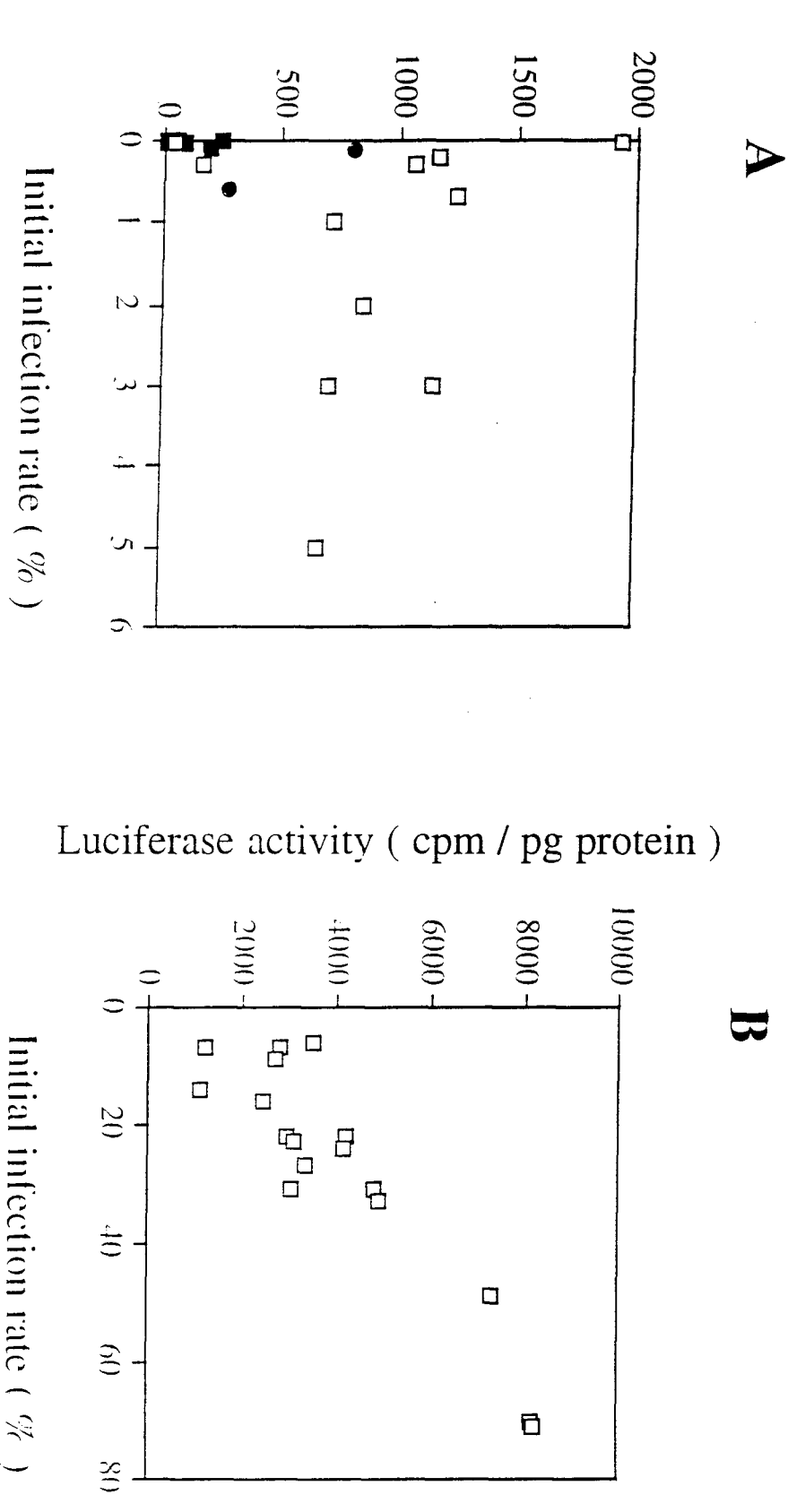

Luciferase activity (cpm / pg protein)
0
500
1000
1500
2000
Initial infection rate (%)
0
1
2
3
4
5
6
A
Luciferase activity (cpm / pg protein)
0
2000
4000
6000
8000
10000
Initial infection rate (%)
0
20
40
60
80
B

Figure 4

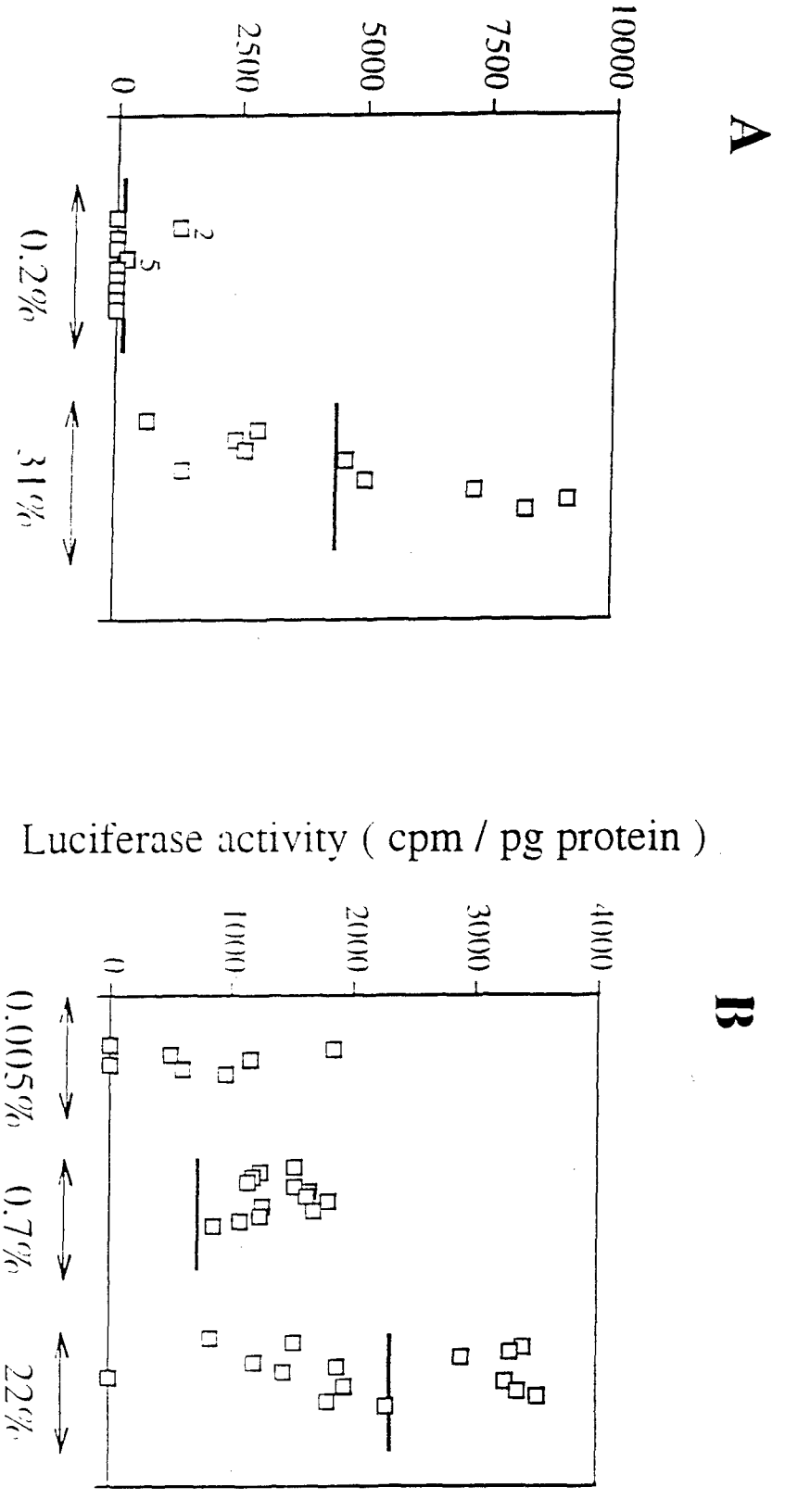

Luciferase activity (cpm / pg protein)
0
2500
5000
7500
10000
0.2%
31%
A
Luciferase activity (cpm / pg protein)
0
1000
2000
3000
4000
0.005%
0.7%
22%
B

Figure 5

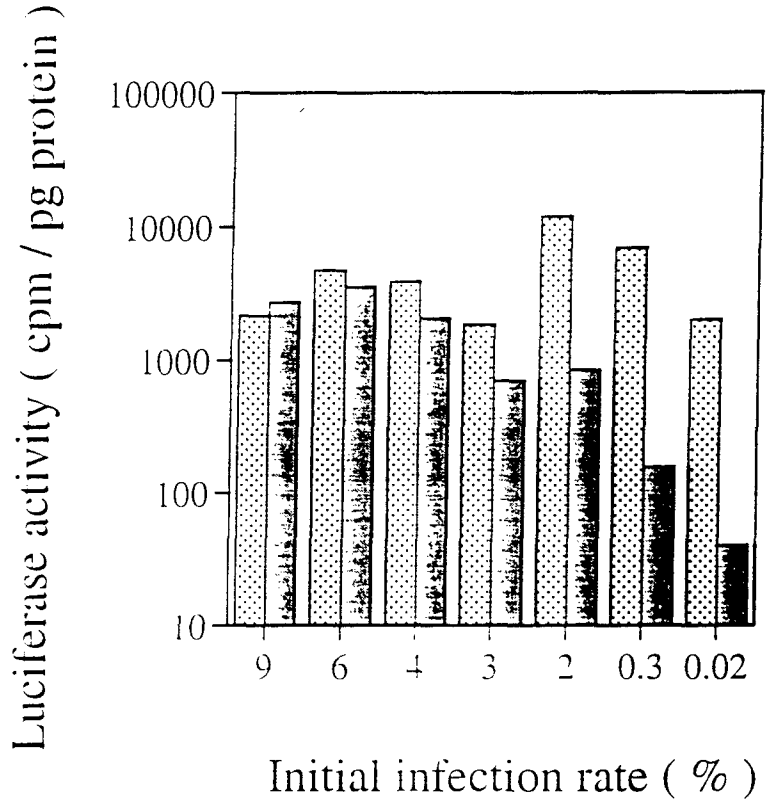
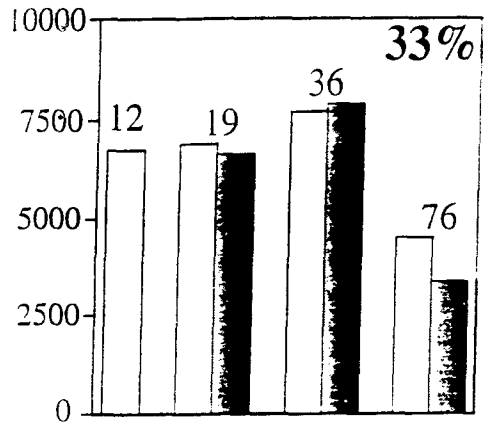
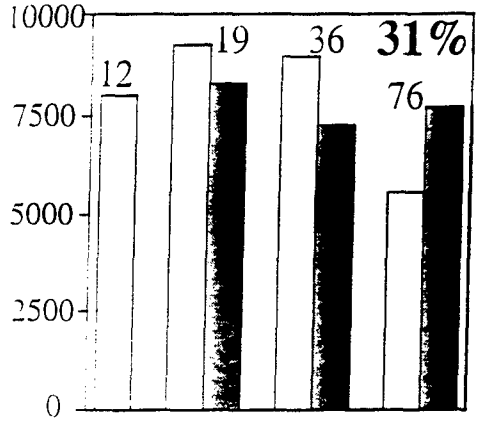
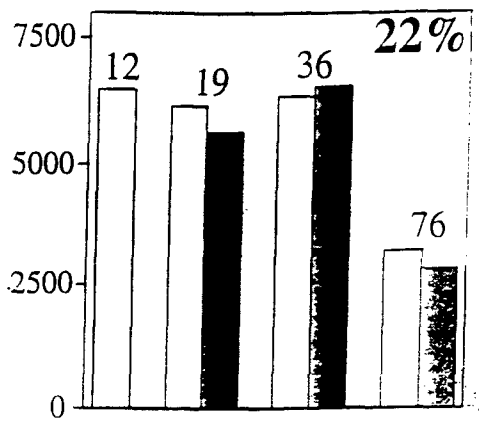
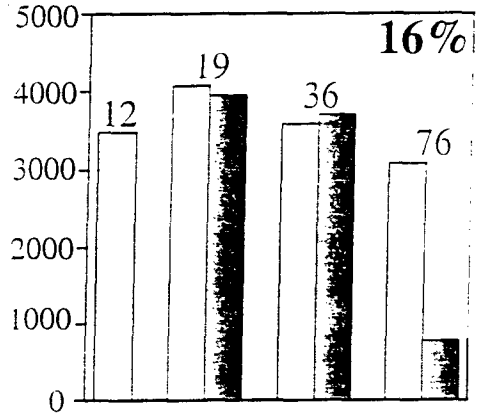

Luciferase activity (cpm / pg protein)
100000
10000
1000
100
10
9
6
4
3
2
0.3
0.02
Initial infection rate (%)

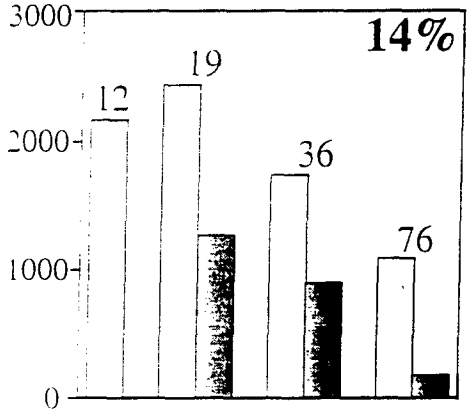
Figure 6

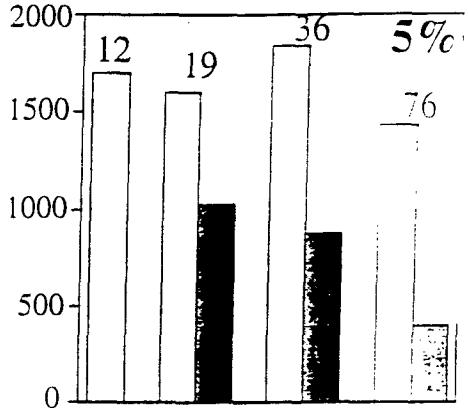

33%
10000
7500
5000
2500
0
12
19
36
76

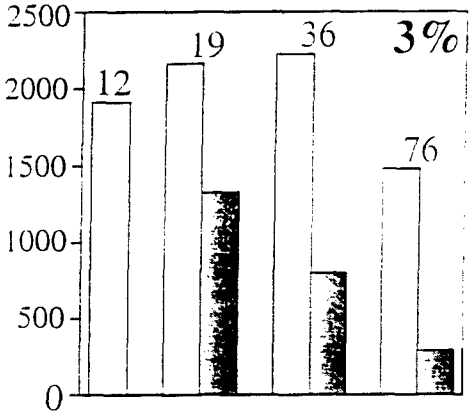

31%
10000
7500
5000
2500
0
12
19
36
76

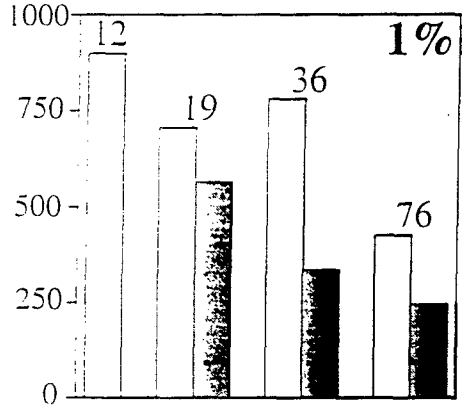

22%
7500
5000
2500
0
12
19
36
76

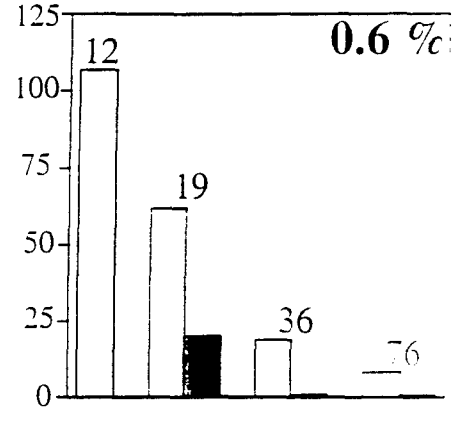

16%
5000
4000
3000
2000
1000
0
12
19
36
76

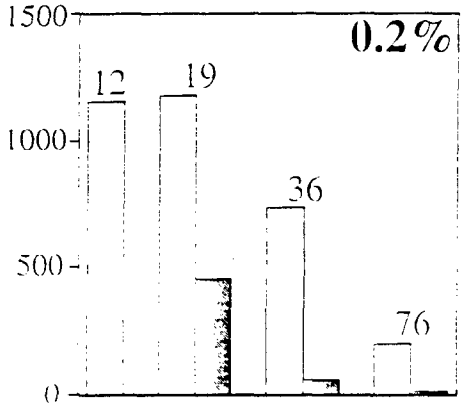

14%
3000
2000
1000
0
12
19
36
76


5%
2000
1500
1000
500
0
12
19
36
76


3%
2500
2000
1500
1000
500
0
12
19
36
76


1%
1000
750
500
250
0
12
19
36
76


0.6 %
125
100
75
50
25
0
12
19
36
76


0.2%
1500
1000
500
0
12
19
36
76

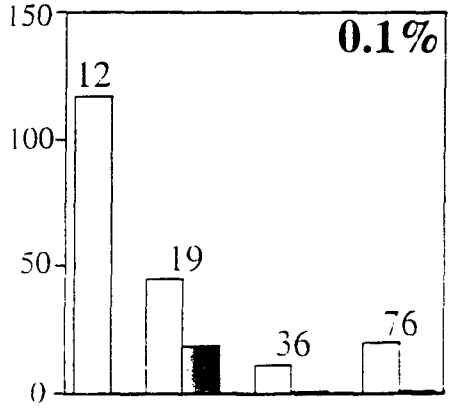
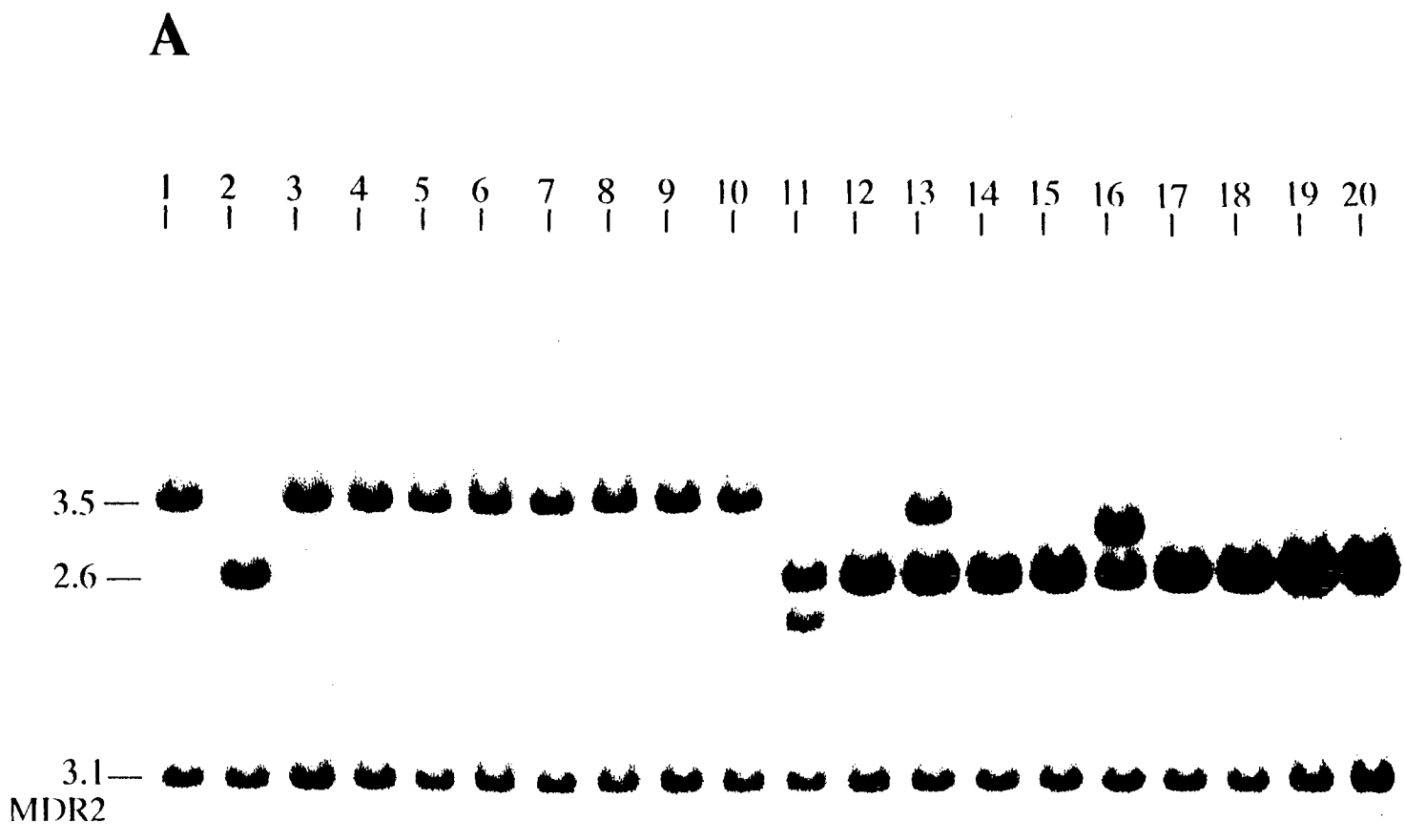

0.1%
150
100
50
0
12
19
36
76

Figure 7a



B

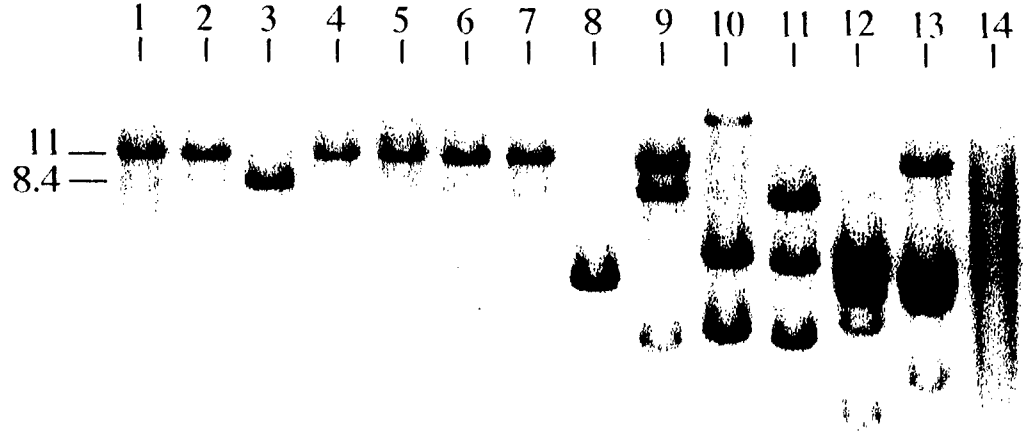


Figure 7b

Figure 7c

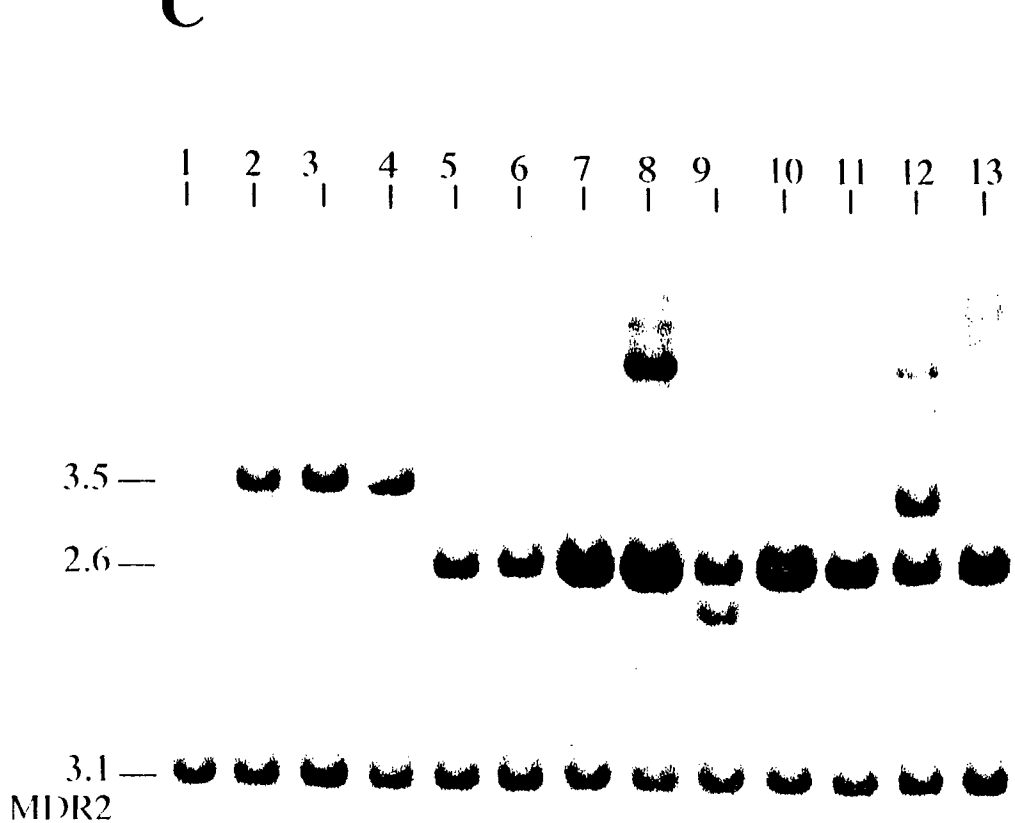


Figure 7d

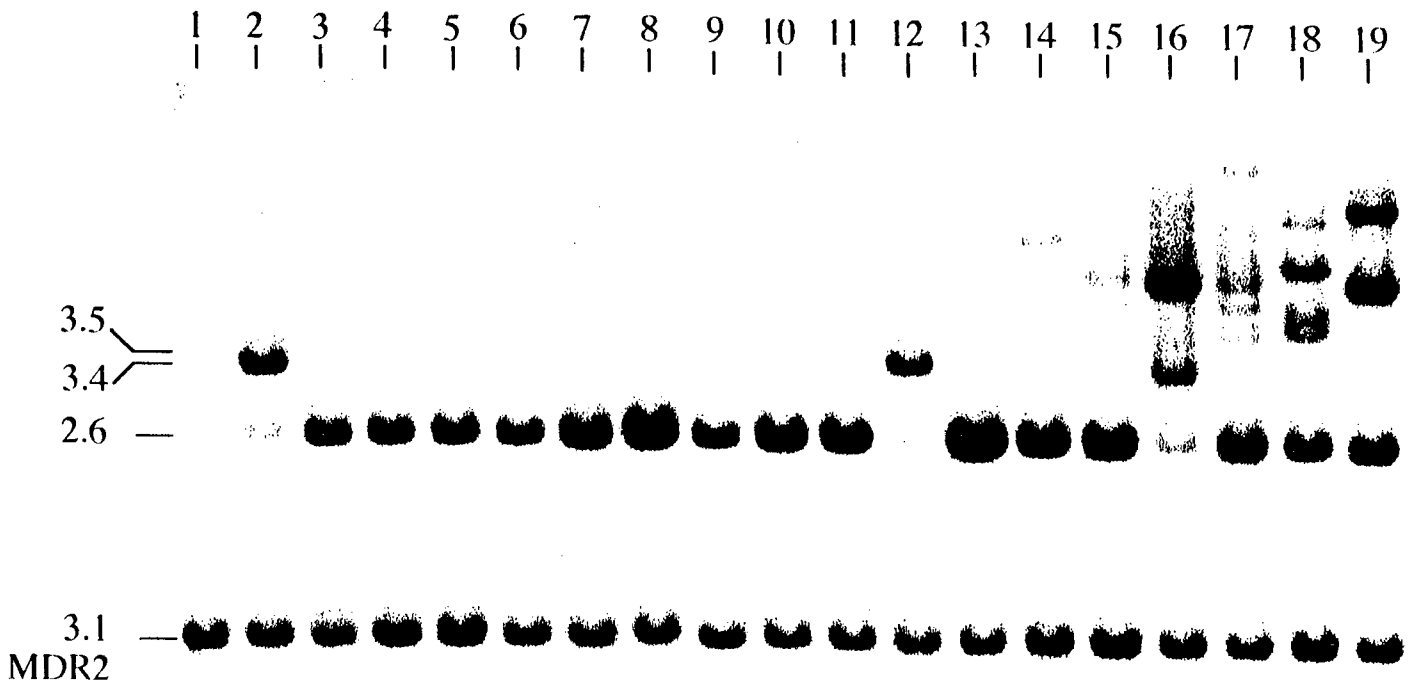


Figure 8

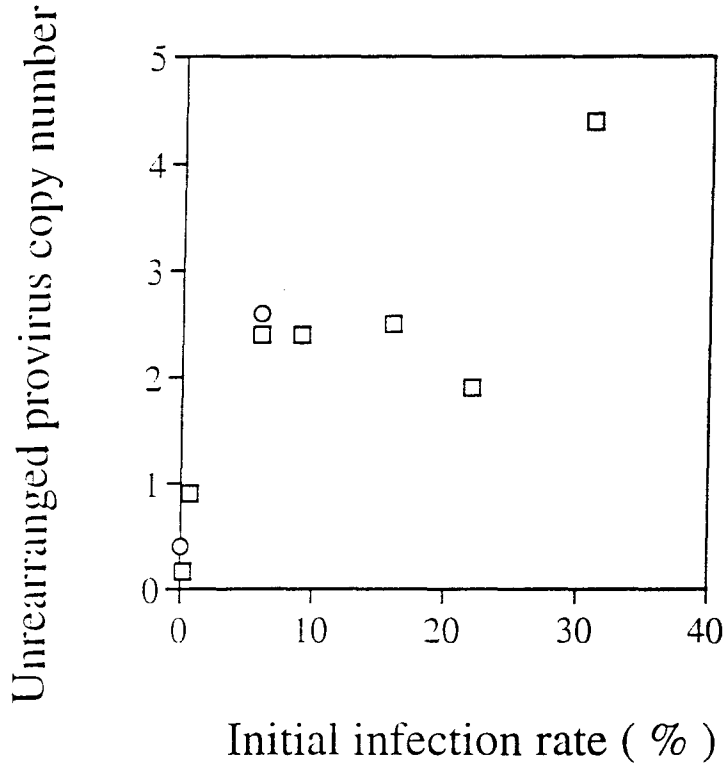

Unrearranged provirus copy number
5
4
3
2
1
0
0
10
20
30
40
Initial infection rate (%)

Figure 9

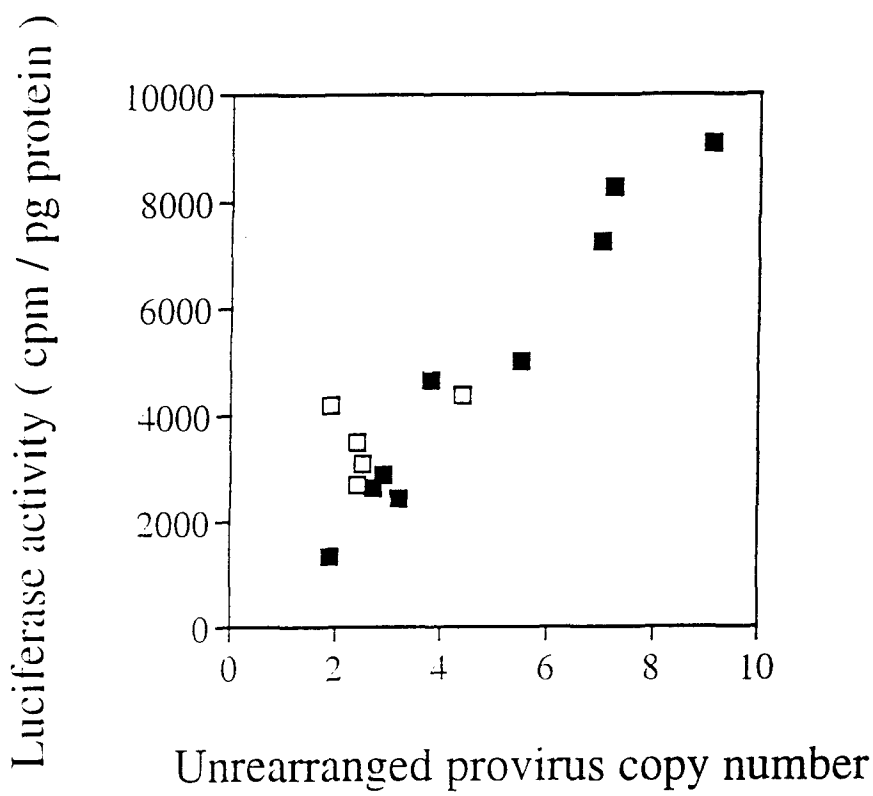
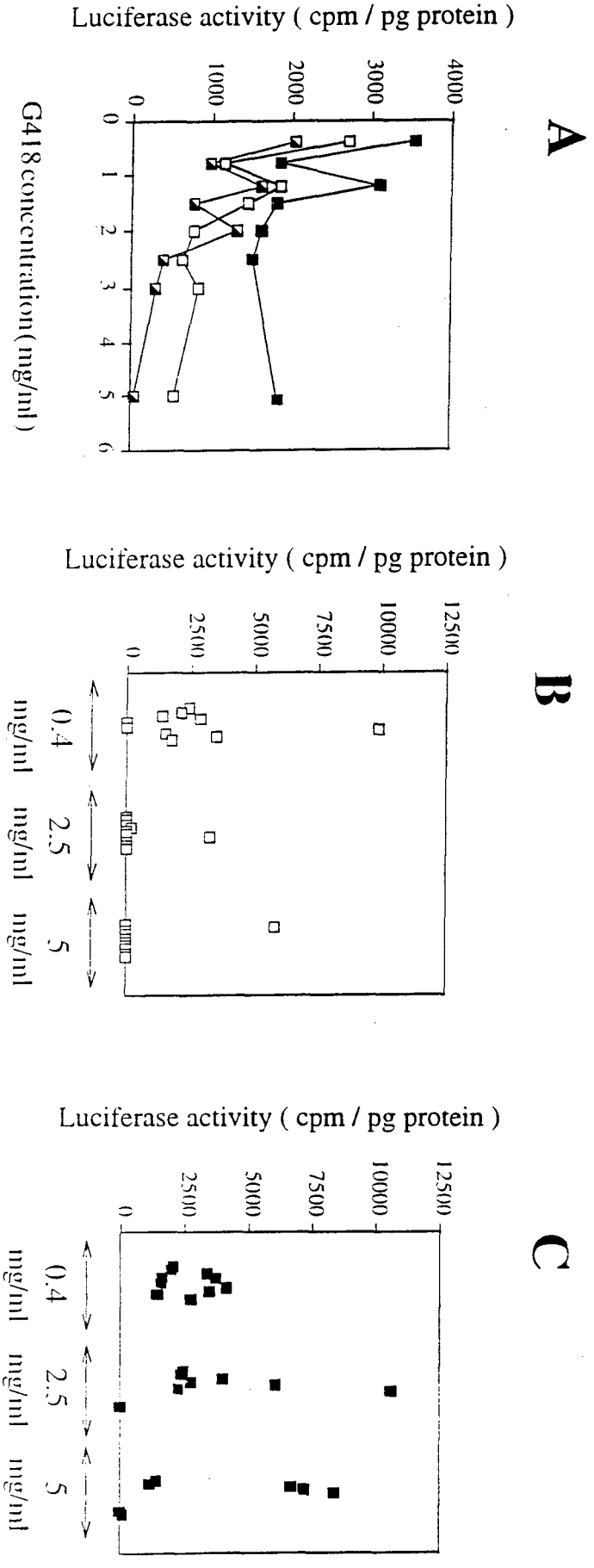

Luciferase activity (cpm / pg protein)
10000
8000
6000
4000
2000
0
0
2
4
6
8
10
Unrearranged provirus copy number

Figure 10


A
Luciferase activity (cpm / pg protein)
0
1000
2000
3000
4000
0
1
2
3
4
5
6
G418 concentration (mg/ml)
B
Luciferase activity (cpm / pg protein)
0
2500
5000
7500
10000
12500
0.4 mg/ml
2.5 mg/ml
5 mg/ml
C
Luciferase activity (cpm / pg protein)
0
2500
5000
7500
10000
12500
0.4 mg/ml
2.5 mg/ml
5 mg/ml

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 3 Mar 98

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCP, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Grant DAMD17-94-J-4038. Request the limited distribution statement for Accession Document Number ADB215795 be changed to "Approved for public release; distribution unlimited." This report should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or email: judy_pawlus@ftdetrck-ccmail.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Completed
2-8-2000
BW