Inducible Gene Expression by DNA Rearrangements in Human Cells

JOHN P. MURNANE

Laboratory of Radiobiology and Environmental Health, University of California, San Francisco, California 94143

Received 25 June 1985/Accepted 23 October 1985

A permanent human cell line, cell line LM205, was established by transforming primary human fibroblasts with a plasmid containing both simian virus 40 sequences with a defective origin of replication and a G418 resistance gene (neo) that lacked a eucaryotic transcriptional promoter. G418-resistant cells appeared spontaneously in clonal populations of LM205 cells at a frequency of approximately $10^{-5}$ cell per cell plated in the presence of 400 µg of G418 per ml. G418 resistance was stable and correlated with the appearance of neo-specific RNA. Characterization of the neo gene in the G418-sensitive parental cell line by both a Southern blot analysis and a restriction map analysis of cloned sequences demonstrated that there was a stable integration site containing a single neo coding sequence. A Southern blot analysis of five G418-resistant subclones indicated that there were heterogeneous DNA rearrangements in the region of the neo gene that were unique in each subclone. Restriction mapping of a fragment containing the neo gene isolated from one of the resistant subclones demonstrated that the rearrangement was a tandem duplication that resulted in the relocation of the simian virus 40 bidirectional transcriptional promoter 5' to the neo gene. Tandem duplication was also consistent with the Southern blot polymorphisms observed in the other resistant subclones, suggesting that there were heterogeneous sites of recombination with respect to both the neo gene and the simian virus 40 promoter. Although these rearrangements resulted in an increase in neo gene copy number per cell, amplification showed no correlation quantitatively with the large increase in neo-specific RNA in these cells. Therefore, G418-resistant colony formation in cell line LM205 provides a method for studying both the mechanisms involved in this type of recombination and the factors influencing its frequency.

DNA rearrangements are responsible for a wide variety of phenomena in mammalian cells, including amplification of genes conferring drug resistance (30, 36) and translocations and transpositions that alter the expression of proto-oncogenes in cancer cells (18, 39). Despite an abundance of information, much remains to be learned about the actual steps in these processes. A model system involving the expression of a single marker gene altered by DNA rearrangements that allows for colony growth in selective medium would be highly useful for studying recombination in mammalian cells. However, investigations into the mechanisms by which mouse cells influence the expression of selectable gene markers have provided the following widely differing results, depending on the nature of the integrated sequences: (i) amplification is responsible for the increased expression of genes that are initially transcribed but partially defective (15, 28); (ii) epigenetic factors influence the expression of intact but repressed genes (7, 9); and (iii) homologous recombination is capable of complementing regulatory or structural defects in two closely linked copies of the same gene having mutations in different locations (20, 21, 33, 37).

To develop a model system to measure gene activation by DNA rearrangements in human cells, I established human cell clones containing the selectable gene marker neo, which was initially inactive because of the absence of a eucaryotic transcriptional promoter. neo is a bacterial gene for neomycin resistance (6) that can be expressed in eucaryotic cells when they are supplied with 5' and 3' eucaryotic regulatory sequences (3, 35). neo can be used for selection of eucaryotic cells because its gene product inactivates the antibiotic G418, which is toxic to eucaryotic cells (5), and because there is no spontaneous resistance to G418 in eucaryotic cells (3, 35). The bacterial derivation of neo decreases the possibility of low-level expression of the promoterless gene and consequent amplification during selection, which have been observed in similar studies in which eucaryotic genes were used (15, 28). However, integration of the neo sequences in the vicinity of host transcriptional regulatory sequences might still produce a clone in which increased transcription could be achieved by amplification or point mutations involving adjacent host cell promoters, enhancers, or termination codons. Despite these possibilities, the appearance of G418-resistant colonies in some clones should indicate the frequency with which active transcriptional promoters have been moved 5' to the integrated neo gene. DNA rearrangements of this type could occur by a number of different mechanisms, including (i) translocation of the neo gene to a new location (18, 39), (ii) transposition of DNA elements, such as interspersed repetitive sequences, into the region of the neo gene (8, 31), and (iii) local recombination events, such as tandem duplications (20, 21, 25, 29, 33, 37). To distinguish among these possibilities, I began to characterize the DNA rearrangements in the region of the integrated neo gene in G418-resistant subclones.

MATERIALS AND METHODS

Cell culture. Normal primary human skin fibroblasts (cell line HS-27) were obtained from the Cell Culture Facility, University of California, San Francisco. Simian virus 40 (SV40)-transformed human skin fibroblasts (cell line GM637) were obtained from James E. Cleaver. All stock cultures were maintained in minimal essential medium (GIBCO Laboratories) containing 10% fetal calf serum (GIBCO) but no antibiotics. For experiments, cells were incubated with 50 µg of gentamicin (GIBCO) per ml. The cells were maintained in tissue culture flasks (Falcon) at 37°C in a humidified incubator containing 5% CO₂.
structure of pSV2neo [35]), minipreparations of plasmid clones were screened by EcoRI digestion to identify plasmids with 4.3- and 6.6-kilobase (kb) fragments (Fig. 1). Restriction mapping was performed to ensure the identity of the pLR309 construction.

**Cell transfection.** pLR309 was cleaved with BglII, which produces a free 5' end of the neo gene which is identical to the end used to construct expression vector pAG60 (3). The plasmid (5 μg) was then introduced by using calcium phosphate precipitation (40) without carrier DNA into 10^6 HS-27 primary human skin fibroblasts. Cells transfected with the plasmid were identified as morphologically transformed foci (26, 32).

Transfection with pSV2neo was carried out as described previously (40). The cells were allowed 3 days for expression of the neo gene and then were plated at a density of 2 × 10^3 cells per 100-mm culture dish in the presence of various concentrations of G418 (35).

**Development of SV40-transformed cell lines.** Morphologically transformed foci were selected by ring isolation (26). After continuous passage in culture for approximately 6 months, human fibroblasts transformed with pSV ori- or pLR309 entered a crisis phase similar to that of SV40 virion-transformed cells (13). By maintaining cells at a high density with regular changes of medium during this period, permanent cell lines (13, 26) were established from four of the pLR309-transformed clones. These cells have now been in culture for more than 2 years. The cells were transferred at low densities to increase plating efficiency and they were clonally selected by ring isolation of low-density colonies.

**Colony formation and growth assays in the presence of G418.** G418-resistant colonies were selected by trypsinizing cells, counting the cells, and plating 2 × 10^5 cells per 100-mm tissue culture dish in medium containing various concentrations of G418. The cells were then incubated at 37°C for 18 to 24 days with changes of medium every 6 days. Plating efficiency was determined by simultaneously plating 2,000 cells in medium without G418.

**Sensitivity to G418 was determined by plating 2 × 10^4 cells in 35-mm tissue culture dishes in growth medium without G418.** The next day, 2-ml portions of growth medium containing various concentrations of G418 were added to duplicate dishes. The cells were then incubated with a change of medium after 4 days. The cells were then trypsinized and counted with a Coulter counter. Because of the variability in activity among different lots of G418, this procedure was used routinely to standardize the concentration of G418 used in these studies.

**Cellular DNA and RNA purification.** Total cellular DNA was prepared as previously described (26). Cytoplasmic RNA was prepared by the method of Favaloro et al. (10), using vanadyl-ribonucleoside complexes (Bethesda Research Laboratories) to inactivate RNase.

**Southern and dot blot analyses.** Digestion of DNA with restriction enzymes and Southern blot analysis (34) were carried out as previously described (26). For the dot blot analysis, DNA samples were denatured by heating at 100°C for 10 min and were diluted serially in 10 mM Tris (pH 7.5)-1 mM EDTA buffer. After an equal volume of 20× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) was added, the samples were filtered through a nitrocellulose filter.

RNA samples were suspended in 10 mM Tris (pH 7.5)-1 mM EDTA buffer and serially diluted, and 0.5 volume of a mixture containing 24% formaldehyde, 0.28 M EDTA, and 0.28 M phosphate buffer (pH 6.8) was added to each sample.

---

**Enzymes and plasmids.** Plasmid pSV ori- was provided by Harvey Ozer (City University of New York), and plasmid pSV2neo was provided by Paul Berg (Stanford University). Plasmid pNeo was purchased from P-L Biochemicals, Inc. Restriction enzymes and bacterial alkaline phosphatase were purchased from Bethesda Research Laboratories. Colicin E1 was kindly provided by Celis Kayalar (University of California, Berkeley). G418 (Geneticin) was purchased from Gibco. RNase A, DNase I, and proteinase K were purchased from Sigma Chemical Co.

**Plasmid construction.** Plasmid pLR309 was constructed from plasmids pNeo and pSV ori- (Fig. 1). The plasmids were prepared as described previously (14) and were purified by cesium chloride-ethidium bromide density centrifugation. Both plasmids were cleaved with BamHI, which cuts pNeo at the 3' end of the neo sequence and separates the pSV ori- sequences from pMK16. The cleaved pNeo fragment was treated with alkaline phosphatase and ligated to the cleaved pSV ori- fragments at a concentration of 0.003 μg/ml with a 2:1 pNeo excess. Ampicillin-resistant colonies were selected after transfection of Escherichia coli HB101 to select for circularized plasmids with pNeo sequences, followed by elimination of colicin E1-positive clones containing pMK16. To select plasmids with the 3' end of the neo gene adjacent to the SV40 late gene termination sequences (similar to the 3'...
The samples were heated at 60°C for 10 min, and 3 volumes of 20× SSC was added to each. The samples were then filtered through a nitrocellulose filter.

Nucleic acid hybridization and probe preparation. After the filters were baked at 80°C for 2 h under a vacuum, they were wetted in 6× SSC and placed in sealable plastic bags in prehybridization solution containing 50% formamide (Bethesda Research Laboratories), 5× SSC, 5× Denhardt solution (1 g of Ficoll per liter, 1 g of polyvinylpyrrolidone per liter, 1 g of bovine serum albumin per liter), 250 μg of denatured sonicated salmon sperm DNA (Sigma) per ml, 20 mM phosphate buffer (pH 6.8), and 0.05% sodium dodecyl sulfate. The filters were incubated in this solution at 42°C for 4 h with constant agitation. The prehybridization solution was removed, and a similar hybridization solution containing 10% dextran sulfate (Pharmacia), 0.1% sodium dodecyl sulfate, and 10 ng of denatured DNA probe (2 × 10^9 to 8 × 10^8 cpm/μg) nick translated with [α-32P]dCTP (>7.000 Ci/mmol; Amersham Corp.) per ml (23, 27) was added. Hybridization was carried out at 42°C for 18 h. The nitrocellulose paper was washed three times for 30 min in 2× SSC containing 0.5% sodium dodecyl sulfate at room temperature and then was washed twice for 30 min in 0.1× SSC containing 0.2% sodium dodecyl sulfate at 50°C. The filter paper was rinsed in 0.1× SSC and dried at room temperature; this was followed by wrapping in plastic wrap and exposure to X-Omat AR film (Eastman Kodak) with Cronex intensifying screens (Du Font Co.) for 12 to 24 h.

neo-specific sequences were prepared by digestion of pNeo to completion with BamHI and HindIII, followed by agarose gel electrophoresis. The 1.5-kb neo fragment was then separated from the 4.0-kb pBR322 fragment by excising the region of the gel containing the neo fragments. The agarose gel slice was placed in dialysis tubing, and the neo fragment was eluted by electrophoresis (24). After the gel slice was removed, the DNA was then dialyzed against 0.5 M NaCl-10 mM Tris (pH 7.2)–1 mM EDTA, separated on a NACS column (Bethesda Research Laboratories), and precipitated in ethanol.

Cloning and analysis of integrated sequences. After complete digestion of genomic DNA with the appropriate restriction enzymes, DNA was electrophoresed as described previously (34). DNA fragments in the size range containing the desired sequences were then sliced from the gel and purified as described above for neo probe preparation. After alkaline phosphatase treatment, the fragments were ligated to a twofold excess of EMBL-4 vector DNA (Promega Biotec) that had been previously digested with the appropriate restriction enzymes (SalI and BamHI for BglII insertions; BamHI and EcoRI for EcoRI insertions) and precipitated with isopropl alcohol (12). The resulting concatamers were then packaged into bacteriophages (Promega Biotec), and the bacteriophage library was screened by hybridization (2), using nick-translated plasmid pLR309. The cloned bacteriophage was then grown and purified by using glycerol step gradients (38) and was analyzed by digestion with various restriction enzymes. Multiple cloned DNA fragments of each type were isolated and compared to eliminate any recombinant variation that might have arisen during bacteriophage cultivation.

RESULTS

Isolation of cell clones. Plasmid pLR309 contains both the origin of replication-defective SV40 transformation sequences and the bacterial neo gene, which were derived from plasmids pSV ori- and pNeo, respectively (Fig. 1).

pLR309 was transfected into primary human fibroblasts by calcium phosphate precipitation without carrier DNA. Twelve individual morphologically transformed foci resulting from integration of SV40 transformation sequences (32) were selected by ring isolation. The plasmid sequences contained in these clones were characterized by Southern blot analysis with a pLR309 probe after digestion with restriction enzymes BglII, SstI, and XbaI, which do not cut linearized pLR309. Unique integration patterns of pLR309 were observed in each of the clones, with one to three separate integration sites (Fig. 2). The labeled bands in the various clones were not much larger than the full-length plasmid and were generally similar to one another in hybridization intensity, although bands smaller than the full-length plasmid were relatively weaker in intensity. These results suggest that little tandem integration or amplification of the individual integrated sequences had occurred, which is consistent with hybridization to known quantities of plasmid DNA (Fig. 2, lane pl). Clone LM205 produced three fragments containing plasmid sequences when it was cleaved with SstI, XbaI, or BglII (Fig. 2); however, one of the BglII fragments was too small (<2 kb) to be observed on this gel. Similar experiments with undigested DNA produced no labeled bands, and therefore fewer than one unintegrated pLR309 plasmid per 100 cells was present in any of these clones. A Southern blot analysis of the integrated plasmid sequences in the permanent lines derived from these initial clones showed that there was no change in the integration pattern.

G418 resistance. To establish whether any of the pLR309-transformed cell clones was capable of expressing the
sensitive of pLR309-transformed subclones RS-2 promoterless nec gene, I tissue culture appearance of 0418-resistant cells in 200 to 400 nonpermanent clone, at which grew from have an effect, as shown by differences in the appearance of LM217 and of other 3) was slightly different from the appearance of LM216 of 400 0418-resistant cell colonies, Only a small percentage of cell line LM205) produced 0418-resistant colonies. Only a few of various 0418-resistant subclones produced 0418-resistant colonies. Only a small percentage of cell line LM205 selected in the presence of 400 µg of G418 per ml. The cells were grown for 8 days.

Characterization of G418 resistance. SV40-transformed human cells that do not contain the neo gene (cell lines LM217 and GM637) or that contain nonfunctional neo gene sequences (cell line LM216) showed variability in G418 sensitivity (Fig. 3 and 4). This variability was associated with differences in the threshold levels at which G418 began to have an effect, as shown by shoulders on the growth curves; the slopes of growth inhibition curves were similar. However, the slope of the growth curve of cell line LM205 (Fig. 3) was slightly different from the slopes of the growth curves of other transformed cell lines, possibly because there was some degree of G418 resistance in the cells in the LM205 population. Colonies of cell line LM205 selected in the presence of 400 µg of G418 per ml showed a large increase in G418 resistance compared with the parental clone (Fig. 3). Colonies selected in the presence of 200 µg of G418 per ml were more frequent and were generally less resistant to G418 than those selected in the presence of 400 µg/ml. The extent of G418 resistance in the subclones selected in the presence of 400 µg of G418 per ml varied but was highly stable, as indicated by the fact that after initial colony isolation in the presence of G418, these clones were grown for approximately 6 weeks without G418 before they were tested for G418 resistance (Fig. 3). Subsequent tests continued to show G418 resistance in these cells.

The extent and variability of G418 resistance in subclones of cell line LM205 selected in the presence of 400 µg of G418 per ml were similar to the extent and variability of resistance in G418-resistant subclones of cell line GM637 transfected with pSV2neo (Fig. 4). The neo gene in the pSV2 expression vector is under the regulation of a strong SV40 transcriptional promoter (35). Increasing the concentration of G418 in the selection medium after pSV2neo transfection reduced the frequency of G418-resistant colonies of cell line GM637. Colonies isolated in the presence of 100 µg of G418 per ml were generally much less resistant than colonies isolated in the presence of 400 µg/ml (Fig. 4), despite the fact that each of the clones contained a single copy of the neo gene.

neo RNA analysis. To ensure that G418 resistance was
associated with neo gene expression, I assayed RNAs from various clones by using dot blot analysis for neo-specific RNA sequences. The DNA probe used in this experiment was the neo gene fragment isolated from agarose gels after BamHI-HindIII cleavage of pNeo. I observed substantially increased hybridization of neo-specific sequences to the RNAs of all of the cell line LM205 G418-resistant subclones compared with either a cell line that does not contain neo (cell line GM637) or the original parental cell line LM205 (Fig. 5). Colonies isolated in the presence of 400 μg of G418 per ml generally showed much higher levels of neo RNA expression than colonies isolated in the presence of 200 μg/ml (Fig. 5), which is consistent with the degree of G418 resistance observed in these cells (Fig. 3). Parental G418-sensitive cell line LM205 showed a small but reproducible content of neo RNA sequences (Fig. 5) as a result of some degree of expression of the neo gene in some or all of the cells in the population. The degree of expression of neo RNA in the G418-resistant subclones of cell line LM205 was comparable to the degree of expression observed in the pSV2neo-transfected GM637 cells selected in the presence of similar concentrations of G418 (Fig. 5).

**Analysis of integrated neo sequences in clone LM205.** To determine whether G418 resistance was accompanied by DNA rearrangements in the region of the neo gene, I performed a Southern blot analysis of integrated neo sequences in cell line LM205 by using the neo fragment isolated from pNeo as a probe. BglII or SstI digestion of DNAs from parental cell line LM205 and six randomly chosen G418-sensitive subclones derived from cell line LM205 (Fig. 6) demonstrated the presence of two fragments containing neo sequences that corresponded to two of the three integrated plasmid sequences shown in Fig. 2. However, only the larger BglII (14-kb) and SstI (20-kb) restriction fragments hybridized sufficiently compared with plasmid standards to suggest that an intact neo gene was present; the sizes of the two smaller restriction fragments indicated that large deletions had occurred in the full-length linear 11-kb plasmid before integration at these sites.

The similarity of the integrated sequences in the cell line LM205 subclones indicated the existence of relatively stable integration sites. The minor additional band at an increased molecular weight observed in parental cell line LM205 after BglII digestion (Fig. 6, lane A) apparently arose after the cloning of this cell line, because it was not seen in earlier clone LM205 populations (Fig. 2) or in the subclones derived from this parental line (Fig. 6, lanes B through G).

In contrast to the G418-sensitive subclones of cell line LM205, a Southern blot analysis of G418-resistant subclones of cell line LM205 digested separately by four restriction enzymes (SstI, XbaI, BglII, and EcoRI) showed that the DNA had been rearranged in the region of the intact integrated neo sequence (Fig. 7). Although G418-resistant colonies selected in the presence of 200 μg of G418 per ml (Fig. 7, lane B) often showed little or no polymorphism in the region of the intact integrated neo gene sequence, each of the five more resistant subclones (selected in the presence of 400 μg of G418 per ml) had additional DNA fragments containing

**FIG. 5.** Dot blot analysis of neo sequences in cytoplasmic RNA preparations from parental cell line LM205 (lane A), cell line LM205 G418-resistant subclone RS-1 (lane B) selected in the presence of 200 μg of G418 per ml, and cell line LM205 G418-resistant subclones RS-2 (lane C), RS-3 (lane D), RS-4 (lane E), RS-5 (lane F), and RS-6 (lane G) selected in the presence of 400 μg of G418 per ml. For comparison, the results obtained with cell line GM637 (lane H), which lacks neo-specific sequences, and with cell line GM637 G418-resistant subclones transfected with pSV2neo and selected in the presence of 100 μg of G418 per ml (subclone R-1) (lane I) or in the presence of 400 μg of G418 per ml (subclone VR-2) (lane J) are shown. The quantity (in micrograms) of RNA added to each well is shown on the right.

**FIG. 6.** Southern blot analysis of integrated neo gene sequences in DNAs from parental cell line LM205 (lane A) and six randomly chosen G418-sensitive subclones (lanes B through G) after digestion with BglII (A) or SstI (B). Each of the plLR309 restriction digest markers (lane pl) from BglII (11.0 kb) and HindIII (2.5 kb) digests contained the equivalent of 2 μg of neo gene sequences. The sizes (in kilobases) of HindIII lambda bacteriophage markers are indicated on the right.
the intact integrated neo gene sequence (Fig. 7, lanes C through G). The absence of detectable rearrangements in a second integration site (14 kb in Fig. 7a; 17 kb in Fig. 7b, etc.) containing a partial neo fragment in these G418-resistant subclones suggested that this polymorphism was associated specifically with the intact integrated neo sequence.

SstI or XbaI digestion (Fig. 7a and b) demonstrated that the new fragments containing the neo gene were larger than the fragments from the parental clone and were different in each subclone (6 to 40 kb). The sizes of the additional fragments in each subclone were the same, regardless of whether SstI or XbaI was used, indicating that the flanking host sequences containing the restriction sites were conserved and that additional DNA sequences (without SstI or XbaI sites) were present within the larger restriction fragment containing the neo gene (20 kb for both SstI and XbaI). There were sometimes multiple rearrangements in a single clone; although these new rearranged bands generally had an intensity comparable to that of the original 20-kb band, there were also lighter bands. These lighter bands may have represented DNA rearrangements containing only partial fragments of the neo gene or may have involved rearrangements that occurred after cloning and were contained in only a fraction of the total population.

In only one of the G418-resistant subclones did the original SstI and XbaI 20-kb neo fragment disappear (Fig. 7a and b, lane C); in three other subclones it was reduced in intensity by approximately 50% (lanes D through F). These results are consistent with the interpretation that G418-sensitive parental cell line LM205 contained two identical copies (i.e., alleles) of this integrated plasmid and surrounding host cell sequences. Therefore, duplication of the entire chromosome or chromosomal region containing the 20-kb SstI or XbaI restriction fragments appears to have occurred after the initial integration of the plasmid in clone LM205. This is a likely possibility, because after transformation of the primary fibroblasts by pLR309, the chromosome number increased from 46 to more than 70 in these cells. My results also indicated that the two copies of this integrated sequence were capable of rearranging independently of each other, as seen in most G418-resistant subclones (Fig. 7a and b, lanes D through G), although in one subclone (lane C) both copies appeared to be rearranged.

A similar analysis of the G418-resistant subclones after BglII or EcoRI digestion indicated that there was further...
heterogeneity in the various DNA rearrangements (Fig. 7c and d). Because these two restriction enzymes produce smaller neo-specific fragments (14 and 6.5 kb, respectively) than SsrI or XbaI, the presence or absence of polymorphism in these fragments in G418-resistant subclones provided additional information on the relative location of the site of recombination. No alteration in banding patterns was observed in subclones RS-2 and RS-4 (Fig. 7, lanes C and E) after EcoRI digestion, indicating that the rearrangements in these two subclones occurred outside the 6.5-kb EcoRI fragment. One of the rearrangements in subclone RS-2 also occurred outside the 14-kb BglII fragment. After BglII digestion, one of the bands appeared to be identical in size to the band seen in the parental cell line; this was confirmed by a restriction map analysis of the cloned BglII fragments (data not shown).

Cloning and analysis of integrated sequences. The integrated plasmid sequences contained in the 14-kb BglII restriction fragment from G418-sensitive parental cell line LM205 were isolated by cloning into bacteriophage vector EMBL-4. A restriction map analysis of several independent isolates demonstrated that nearly the entire linear pLR309 plasmid was integrated at this site and that no tandem plasmid integration was apparent (Fig. 8A).

Isolation and a restriction map analysis of the rearranged 13-kb EcoRI fragment from G418-resistant subclone RS-3 demonstrated that there was tandem duplication in the region of the integration site (Fig. 8B). The single site of recombination between the flanking host sequences (Fig. 8B, large arrow) would have resulted in the complete duplication of the integrated plasmid. This structure is consistent with the changes observed in both the size and the intensity of neo-specific bands by Southern blot analysis (Fig. 7), assuming that a second unrearranged copy of the original integration site was also present in these cells. For example, (i) with SsrI and XbaI, the original 20-kb band would have been reduced in intensity by one-half, to one copy per cell, whereas a new band that was 13 kb larger and contained two neo sequences would have appeared; (ii) with BglII, the intensity of the original 14-kb band would have been unchanged, because the 3' end of the recombination occurred outside this restriction fragment containing one of the dupli-
cated neo genes (however, a new band containing a single neo sequence would have appeared to be 1.5 kb smaller because of the new BgIII site close to the 5' end of the other neo sequence in the duplicated region); (iii) with EcoRI, the intensity of the original 6.5-kb band would have been unchanged because of the recombinational site distal to the EcoRI fragment containing one of the duplicated neo genes, whereas a new band that was 6.5 kb larger would have appeared as a result of recombination near the 5' end of the other duplicated neo gene. Therefore, the cloned EcoRI fragment accurately represented the structure of the DNA in the region of the integrated neo gene in this G418-resistant subclone.

Rearrangements similar to that shown for subclone RS-3 would also be consistent with the Southern blot polymorphism observed for other G418-resistant subclones, assuming that recombination had occurred in each case within the SstI or XbaI fragments but in heterogeneous locations relative to the BgIII and EcoRI restriction sites. An example is shown in Fig. 8C for subclone RS-5. This structure is consistent not only with changes in the sizes of the neo-specific fragments, but also with the fact that, unlike subclone RS-3, the intensity of the rearranged BgIII and EcoRI fragments appeared to indicate that they contained two copies of the neo gene sequence. The size of the new EcoRI fragment (12.5 kb) was nearly twice that of the original fragment (6.5 kb), also indicating that the rearrangement probably occurred 5' to the neo gene sequence.

The structure of the tandem duplication shown for subclone RS-3 and suggested by Southern blot analysis for the other G418-resistant subclones would have duplicated both the neo gene sequence and the SV40-defective origin of replication. Since the SV40 origin contains a strong bidirectional transcriptional promoter, these rearrangements would now place one of the neo sequences under the control of the SV40 late gene promoter. Therefore, large increases in neo RNA expression in the G418-resistant subclones might be expected from these types of rearrangements.

Tandem duplication at the site of integration in the G418-resistant subclones should have resulted in an increase in neo sequence copy number in these cells. This was confirmed by a dot blot analysis (data not shown) and is evident in the Southern blots shown in Fig. 7. However, the small increase in neo gene copy number did not correlate quantitatively with the substantial increase in neo-specific RNA in these cells (10- to 30-fold). Two subclones (subclones RS-3 and RS-5) (Fig. 7, lanes D and F) exhibited only a 50% increase in neo-specific sequences compared with parental cell line LM205; this was consistent with an increase from two copies per cell to three copies per cell. In subclone RS-2 (lane C), there were twice as many neo-specific sequences as in the original cell line; this was consistent with the hypothesis that both original copies had undergone tandem duplication. Amplification progressed much farther in subclone RS-4 (lane E), with a threefold increase in copy number compared with the original parental cell line. These results, combined with the large increase in size of the new bands in this clone (up to 40 kb), may indicate that there were amplified arrays containing several neo gene copies as a result of multiple recombination events.

**DISCUSSION**

Cell line LM205 spontaneously developed G418-resistant subclones that exhibited rearrangements and stable expression of the selectable gene marker neo, which was initially inactive because of the absence of a transcriptional promoter. The absence of detectable DNA rearrangements in another integrated plasmid sequence in the same G418-resistant subclones and the low frequency of rearrangements of neo gene sequences in clones not expressing the neo gene strongly suggest that DNA rearrangements in the region of the neo gene were responsible for expression of the neo gene. Isolation and analysis of the rearranged fragment containing the neo gene sequence in one G418-resistant subclone demonstrated that the observed polymorphism was a result of tandem duplication involving both the integrated plasmid and the adjacent sequences. This duplication event resulted in the relocation of the SV40 bidirectional transcriptional promoter 5' to the neo gene, which could explain the greatly enhanced expression of the neo gene in this subclone. Tandem duplications occurring at heterogeneous sites relative to the neo gene and the SV40 transcriptional promoter would also be consistent with the Southern blot polymorphisms observed in other G418-resistant subclones. Therefore, the DNA rearrangements responsible for G418-resistant colony formation in cell line LM205 appear to be the result of tandem duplications of DNA containing both the neo gene and the SV40 promoter. Further analysis of the factors which influence the frequency of G418-resistant colony formation and the types of DNA rearrangements involved should provide a better understanding of this process.

The tandem duplications in cell line LM205 are similar in many respects to DNA rearrangements involving integrated viral sequences of mouse and human cells transformed with SV40 virus (1, 16, 17, 25, 29). Analyses of the DNA rearrangements in SV40 by Sager et al. (29) and Mounts and Kelly (25) clearly demonstrated that they also resulted from tandem duplication at the site of integration. However, the rate of spontaneous DNA rearrangements of the integrated plasmid sequences in cell line LM205 (Fig. 6) was much lower than the rate generally observed with integrated SV40 sequences in mouse cells (15, 25, 29). Integrated SV40 sequences in subclones of other transformed human cell lines (17) have also shown a high degree of stability, which may be further enhanced in cells transformed by pLR309 by the absence of the SV40 origin of replication required for SV40 sequence amplification (19). Whether SV40 transformation or the presence of integrated sequences has an influence on the frequency of DNA rearrangements in the region of the neo gene is unclear.

My results with cell line LM205 differ in many respects from those of previous studies in which workers investigated the mechanism of enhancement of expression of various selectable gene markers in mouse cells. Roberts and Axel (28) selected cells that spontaneously expressed a promoterless thymidine kinase (tk) gene. However, the clones that expressed the TK+ phenotype had a 20- to 30-fold amplification of the tk gene, which originally showed a low level of transcription because of a second weak promoter in the gene. I used the neo gene to avoid a similar situation, because it is derived from bacteria (6) and lacks any detectable eucaryotic promoter activity (3, 35). Hiscott et al. (15) demonstrated that amplification was also responsible for selection at restrictive temperatures in a cell line containing an SV40 temperature-sensitive A gene. As in the work described here, the amplified DNA sequences demonstrated that there were DNA rearrangements. However, in the work of Hiscott et al. the relatively small increase in gene copy number provided sufficient additional gene activity to compensate for the partially defective A gene product.

In two investigations into the mechanism of induction of
intact tk or neo genes that contained complete eukaryotic transcriptional regulatory sequences workers have concluded that epigenetic factors can also be involved in activation of selectable gene markers (17, 9). Although in my study a slight degree of neo gene expression was observed without detectable DNA rearrangements (clones selected in the presence of 200 μg of G418 per ml), DNA rearrangements were always associated with a high degree of expression of the neo gene.

Several investigators have studied the activation of two closely linked copies of the same selectable gene (tk or neo), each containing a separate mutation in a different location (20, 21, 33, 37). The activation of these genes involved both reciprocal and nonreciprocal homologous recombination. Some of these rearrangements, which were believed to involve unequal sister chromatid exchange, were similar to the recombination events observed in the present study. However, the rearrangement of the neo gene in cell line LM205 may represent a different process, because the variability in the site of recombination in the various G418-resistant subclones, which involved both plasmid and adjacent host sequences (Fig. 7 and 8), does not suggest sequence-specific recombination. Therefore, the rearrangements involving the integrated plasmid in cell line LM205 might involve nonhomologous recombination and, if so, would represent the first step in amplification of nonrepetitive sequences. Nonhomologous recombination would be consistent with the absence of detectable repeated sequences in the single integrated plasmid pLR309. However, the nature of flanking host sequences (e.g., repeat sequences, transposable elements) may influence the expression (11) or the rate (29, 33) of rearrangement of the neo gene. The nature of the host DNA sequences at the integration site may be important in cell line LM205, because it was the only pLR309-transformed cell line that spontaneously expressed the neo gene. Alternatively, cell line LM205 may have been the only clone to obtain an intact neo gene, because some of the original transformed clones had portions of the neo gene deleted from the integrated plasmid (data not shown). Further analysis of the host sequences in the region of the integration site should provide additional information on the role of these sequences in the recombination events observed.

ACKNOWLEDGMENTS
I thank Robert Painter for helpful discussions and support and Mary McKenney and Leslie Roberts for assistance with the manuscript.

This work was supported by contract DE-AC03-76-SF01012 from the U.S. Department of Energy.

LITERATURE CITED


