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Oncogenes Result in Genomic Alterations That Activate a Transcriptionally Silent, Dominantly Selectable Reporter Gene (*neo*)

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Although oncogenes and tumor suppressor genes have been implicated in carcinogenesis and tumor progression, their relationship to the development of genomic instability has not been elucidated. To examine this role, we transfected oncogenes (polyomavirus middle [Py] and large T [MT and LT]) and adenovirus serotype 5 E1A) into two NIH 3T3-derived cell lines, EN/NIH 2-4 and EN/NIH 2-20. Both cell lines contain two stable integrants of a variant of the retrovirus vector pZipNeoSV(x)1 that has been modified by deletion of the enhancer elements from the long terminal repeats. DNA rearrangements activating the silent neomycin phosphotransferase gene (*neo*) present in these integrants were identified by selection of cells in the antibiotic G418. Whereas control-transfected EN/NIH cell lines do not yield G418-resistant subclones (GRSs), a fraction of oncogene-transfected EN/NIH 2-4 (8 of 19 Py MT, 5 of 17 Py LT, and 11 of 19 E1A) and 2-20 (7 of 15 Py MT) cell lines gave rise to GRSs at differing frequencies (0.33×10^{-6} to 46×10^{-6} for line 2-4 versus 0.11×10^{-6} to 1.3×10^{-6} for line 2-20) independent of cell generation time. In contrast, a distinctly smaller fraction of mutant Py MT-transfected EN/NIH cell lines (1 of 10 MT23, 1 of 10 MT1015, and 0 of 10 MT59b) resulted in GRSs. Southern analysis of DNA from selected oncogene-transfected GRSs demonstrated genomic rearrangements of *neo*-containing cellular DNA that varied in type (amplification and/or novel fragments) and frequency depending on the specific oncogene and EN/NIH cell line used in transfection. Furthermore, only one of the two *neo*-containing genomic loci present in both EN/NIH cell lines appeared to be involved in these genomic events. In addition to effects related to the genomic locus, these observations support a role for oncogenes in the development of genetic changes associated with tumor progression.

Oncogenes and tumor suppressor genes are central to the immortalization and transformation of primary cells (16, 27, 28). They are also implicated in oncogenesis and tumor progression, processes which are typically associated with the accumulation of increasing numbers of karyotypic and subchromosomal abnormalities (26, 32–34, 46). These genetic alterations are frequently nonrandom, involving one or more of the same genetic loci in phenotypically and histologically similar tumors obtained from different individuals. For example, a stepwise but not necessarily ordered series of nonrandom genomic rearrangements has been associated with the progression of colorectal adenomas to adenocarcinomas (53). Similarly, increasing numbers of karyotypic abnormalities, in addition to the Philadelphia chromosome, are observed as chronic myelogenous leukemia progresses from the stable phase to the accelerated phase and blast crisis (30). The increased capacity of tumor cells to give rise to the genetic changes which accompany tumor progression has been coined genomic instability and is the property of tumor cells believed to increase the probability that variants within a tumor cell population will arise that have a competitive advantage over their normal counterparts (32–34). It is generally accepted that specific oncogenes and tumor suppressor genes are involved in the genetic events important to the expression of the neoplastic phenotype. However, the relationship of oncogenes or tumor suppressor genes to the development of genomic instability has not been elucidated.

To examine the role of oncogenes in the development of genomic instability, we transfected selected DNA virus oncogenes into two NIH 3T3-derived cell lines, EN/NIH 2-4 and EN/NIH 2-20. Both cell lines contain two stable integrants of a variant of the retrovirus vector pZipNeoSV(x)1 (6) that has been modified by deletion of the enhancer elements from the long terminal repeats (LTRs) and deletion of the 3′ splice acceptor site located between the *Bam*HI site and the neomycin phosphotransferase gene (*neo*) (43) (Fig. 1). Promoter elements in the 5′ and 3′ LTRs are retained in this retrovirus vector (retrovector) variant. This approach was founded on the expectation that DNA rearrangements activating the silent *neo* gene present in these integrants could be identified by selection of cells in the antibiotic G418. Since parental EN/NIH cells do not express *neo* or give rise to G418-resistant subclones (GRSs) when subjected to G418 selection (43), we were able to assess whether specific oncogenes expressed in EN/NIH cell lines would result in a change in the rate of reactivation of the *neo* reporter gene. Additionally, by Southern analysis with a *neo* probe, we identified several types of genetic changes associated with activation of *neo* in randomly selected, independently derived GRSs. In this way, we hoped to obtain insight into the mechanisms by which specific oncogenes might result in activation of a silent-gene locus.

We studied three oncogenes that differ in their cellular sites of action and capacities to transform: the middle T (MT) and large T (LT) genes of polyomavirus (Py) and the E1A gene of adenovirus serotype 5. Both Py MT and E1A have transforming activity in established rodent cell lines, whereas Py LT has an establishment function but does not transform established cells; Py MT antigen (MTAg) acts at the cell membrane, whereas Py LTAg and E1A act in the nucleus (9, 17, 23, 37, 40, 51, 57). Furthermore, in contrast to a temperature-sensitive mutant of the simian virus 40 (SV40) large T (LT) antigen and a tumor promoter (12-*O*-tetrade-

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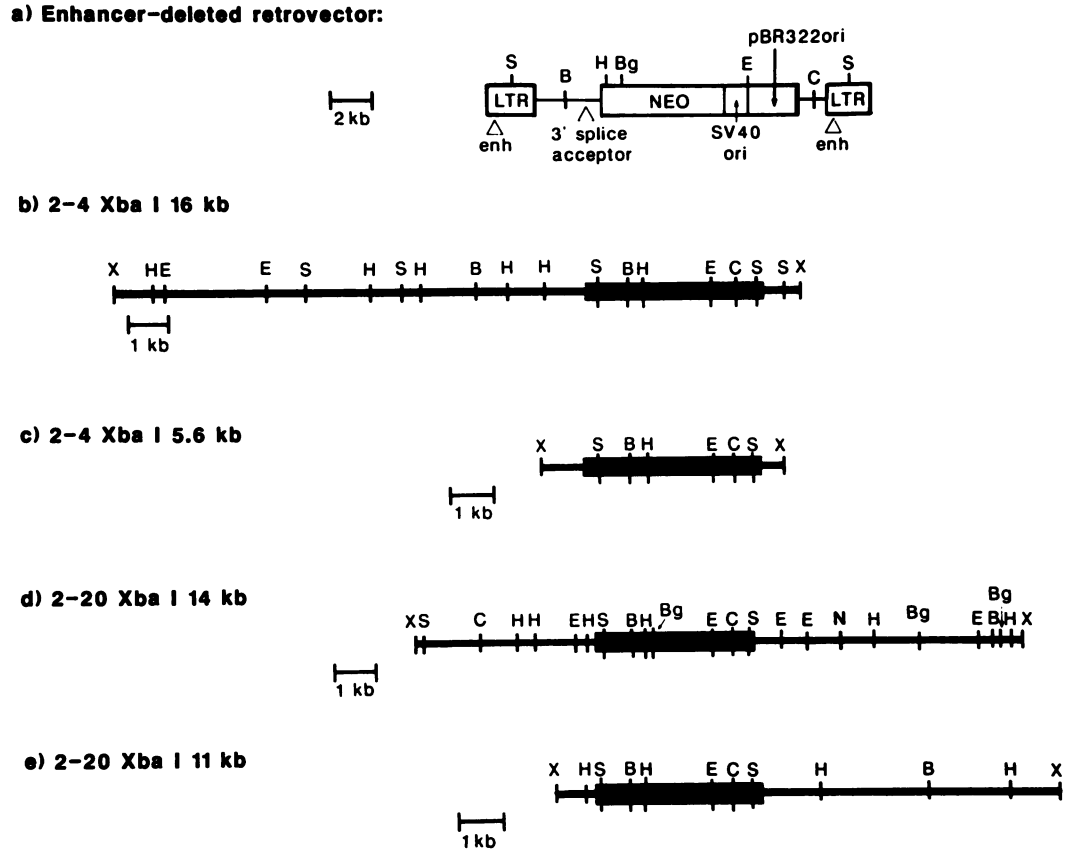

FIG. 1. Restriction enzyme maps of (a) the enhancer-deleted variant of the pZipNeoSV(x)1 retrovector and (b through e) the retrovector-containing *Xba*I fragments isolated from the parental EN/NIH 2-4 and 2-20 cell lines by a previously described protocol (43). Each cell line contains two integrants of the enhancer-deleted retrovector. Thin and thick lines represent cellular and retrovector DNA, respectively. enh, enhancer; ori, origin of replication. Restriction enzymes: X, *Xba*I; S, *Sac*I; C, *Cla*I; E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; Bg, *Bgl*II; N, *Nde*I.

canoylphorbol-13-acetate [TPA]) used in previous work (43), these oncoproteins have no known direct or indirect associations with sequences—in particular, the SV40 origin of replication (*ori*)—present in the EN/NIH *neo*-containing retrovector integrants. In addition, to substantiate observations made with wild-type oncogene, we examined several related mutant oncogenes whose protein products differ in their associated biological and biochemical activities. Because they had been well characterized and constructs expressing the protein products were readily available (31), we compared the effects of wild-type MTAg (MTWT) and three MTAg mutants (MT1015, MT23, and MT59b) defective in the ability to transform rodent fibroblast cells and differing in their associated tyrosine kinase (TK, $pp60^{c\text{-}src}$) and phosphatidylinositol kinase (PIK, p85) activities (31).

MATERIALS AND METHODS

Cell lines and plasmids. Derivation of the EN/NIH cell lines (a gift of Richard C. Mulligan) has been described before (43). The EN/NIH 2-4 and EN/NIH 2-20 cell lines represent two independent subclones that both demonstrated two retrovector integrants by Southern analysis (see Fig. 1b through e, 2, and 3) and absence of *neo* transcripts by Northern (RNA blot) analysis (43).

The plasmids used in transfection are listed in Tables 1 and 2. The pBluescriptSK(+) cloning vector (Stratagene) was used as a negative control. pY3 is a eukaryotic vector which expresses the hygromycin phosphotransferase gene (*hph*) downstream of the promoter and enhancer elements of the Moloney murine sarcoma virus LTR (4). pPyMT and pPyLT

TABLE 1. Fraction of control- and oncogene-transfected EN/NIH 2-4 and EN/NIH 2-20 cell lines yielding G418-resistant colonies and frequencies of GRSs observed

Cell line transfected	DNA used in transfection[a]	No. of subclones yielding $G418^r$ colonies/no. tested	Median (range) frequency[b] (10^{-6})
EN/NIH 2-4	pBluescript+pY3	0/22	—[c]
	pPyMT+pY3	8/19	3.9 (0.67–21)
	pPyLT+pY3	5/17	1.6 (0.67–18)
	pE1A+pY3	11/19	1.2 (0.33–46)
EN/NIH 2-20	pZipHph	0/10	—
	pZip(MTWT)hph	7/15	0.50 (0.11–1.0)
	pZip(MT23)hph	1/10	NA[d] (1.3)
	pZip(MT1015)hph	1/10	NA (0.33)
	pZip(MT59b)hph	0/10	—

[a] For description of plasmids used in transfection, see Materials and Methods.
[b] Per cell placed into G418 selection.
[c] —, $<10^{-8}$ (inferred); see Results for details.
[d] NA, not applicable; number in parentheses is the frequency observed for the single subclone that gave rise to GRSs.

TABLE 2. Summary of Southern blot analyses of DNA from selected GRSs derived from oncogene-transfected EN/NIH 2-4 and 2-20 cell lines[a]

Cell Line transfected	DNA used in transfection	No. of GRSs examined	No. of GRSs with:				
			Amplification	Novel fragment	Both amplification and novel fragment	Larger *Xba*I fragment involved	Smaller *Xba*I fragment involved
EN/NIH 2-4	pPyMT+pY3	29	21	6	5	22	0
	pPyLT+pY3	10	4	1	0	5	0
	pE1A+pY3	25	11	2	2	11	0
EN/NIH 2-20	pZip(MTWT)hph	5	3	5	3	5	0
	pZip(MT23)hph	2	2	2	2	2	0
	pZip(MT1015)hph	1	0	1	0	1	0

[a] All GRSs that were selected and expanded were evaluated for the presence of *neo* amplification, appearance of a novel *neo* fragment, or both. Additionally, the cellular *Xba*I *neo*-containing DNA fragment involved in these genomic events was examined. Restriction enzymes used: *Xba*I, *Sac*I, and *Bam*HI for all samples; additionally, *Eco*RI for EN/NIH 2-20-derived cell lines. Two unique *neo*-containing cellular DNA fragments are defined by *Xba*I digestion of EN/NIH 2-4 and 2-20 DNA (see Fig. 1 through 3).

are polyomavirus-derived plasmids (American Type Culture Collection) which express the MT and LT oncogenes, respectively, from the promoter and enhancer elements of Py (37, 51, 57). pE1A expresses the E1A oncogene derived from adenovirus type 5 (52) and was subcloned by deletion of the 2.3-kbp *neo*-containing *Eco*RI-*Eco*RI fragment from p1Aneo (a gift of Earl Ruley) and ligation of the 4.5-kbp E1A-containing pUC18 backbone. pZipHph expresses *hph* and was derived from pZipNeoSV(x)1 and pY3 by replacing the *neo*-containing *Xho*I-*Xho*I insert of pZipNeoSV(x)1 with the *hph*-containing *Sst*I-*Kpn*I fragment from pY3. pZip(MTWT)hph, pZip(MT23)hph, pZip(MT1015)hph, and pZip(MT59b)hph express the wild-type (MTWT), deletion (MT23 and MT1015), and insertion (MT59b) mutants of MT (31) from the unique retroviral *Bam*HI site of the pZip vector, in addition to *hph*. These plasmids were derived, respectively, from pZip(MTWT)NeoSV1, pZip(MT23)Neo SV1, pZip(MT1015)NeoSV1, and pZip(MT59b)NeoSV1 (31) (gifts of Thomas M. Roberts) by a cloning strategy analogous to that described above for pZipHph. The restriction enzyme DNA digest fragments used in the pZip ligations were blunt-ended with S1 nuclease before ligation with T4 DNA ligase. DNA manipulations were performed by established techniques (1, 29).

Transfections and culture conditions. Transfections of EN/NIH 2-4 and 2-20 cell lines were performed by electroporation (10) under conditions empirically established to yield optimum transfection efficiencies for the NIH 3T3 cell line: 10^6 cells in 350 μl of Dulbecco's minimal essential medium (DME), 330 μF capacitance, 200 V, and low Ω resistance setting on the Cell-Porator (Bethesda Research Laboratories). A total of 20 μg of plasmid DNA was used for each transfection; cotransfections (Tables 1 and 2) were performed with a 9:1 ratio by weight of oncogene-containing plasmid to pY3. Cells were grown on GIBCO tissue culture plates in DME containing 10% bovine calf serum (Hyclone) under a humidified atmosphere containing 5% CO_2 at 37°C. Following electroporation, cells were grown for 48 h in the absence of hygromycin selection pressure and then replated at 20× dilution. After 24 h, hygromycin (150 μg/ml) was added to the medium.

Independent hygromycin-resistant colonies (100 to 200 cells) that emerged after 14 to 21 days in hygromycin selection were randomly selected, individually harvested, expanded (to 10×10^6 to 30×10^6 cells), and subjected to G418 selection (600 μg [total] of G418 per ml) at densities of 0.75×10^6 or 1.5×10^6 cells per 150-mm tissue culture plate, for a total of 3×10^6 and 18×10^6 cells per EN/NIH 2-4- and 2-20-derived hygromycin-resistant cell line, respectively. G418-resistant colonies (>50 cells) that emerged after 21 to 42 days were counted to determine the frequency of GRSs observed per cell placed into selection. Independent GRSs (100 to 200 cells) were randomly selected, individually harvested, and expanded (to 10×10^6 to 30×10^6 cells) in medium containing G418.

Isolation and analysis of cellular DNA. Total cellular DNA (and RNA) were isolated from harvested cells stored at −70°C by lysis in 300 mM sodium acetate–50 mM EDTA (pH 7.5)–hot phenol-chloroform (65°C) and centrifuged through cesium chloride as described previously (7). Subsequent DNA manipulations, including Southern analyses of complete restriction enzyme digests of total cellular DNA (2.5 or 5 μg), were performed by established techniques (29). ^{32}P-labeled DNA probes were prepared with random oligonucleotides as primers (15). The probe for *neo* was the 1.2-kbp *Mst*II-*Hin*dIII fragment of the *neo* gene derived from pSV2Neo (45); probes for the Py LT and wild-type and mutant MT oncogenes were the 1.5-kbp *Bam*HI-*Bam*HI MTWT insert of pZip(MTWT)NeoSV1 or the 5.1-kbp *Bam*HI-*Bam*HI MT-containing fragment of pPyMT; and the probe for E1A was the 1.8-kbp *Pst*I-*Eco*RI fragment of the E1A gene derived from pE1A. Following hybridization, filters were washed to a final stringency of 0.75× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C and autoradiographed on Kodak X-Omat AR film at −70°C.

RESULTS

Oncogene-induced activation of *neo* in EN/NIH 2-4 cells. We first chose to examine the capacity of the Py MT, Py LT, and E1A oncogenes to induce activation of *neo* in EN/NIH 2-4 cells. As detailed in Materials and Methods, randomly selected, independent control- and oncogene-transfected hygromycin-resistant EN/NIH 2-4 cell lines were obtained following cotransfection of EN/NIH 2-4 cells with pY3 and pBluescript, pPyMT, pPyLT, or pE1A. As expected (17, 37, 40, 51, 57), Py MT- and E1A- but not Py LT-transfected EN/NIH 2-4 subclones appeared morphologically transformed. Cellular genomic integration (in each instance, a single copy with an integration site unique for each cell line) and expression of the transfected oncogene were demonstrated in oncogene-transfected EN/NIH 2-4 subclones by Southern and Northern analyses, respectively, using ^{32}P-oligolabeled oncogene-specific probes (data not shown).

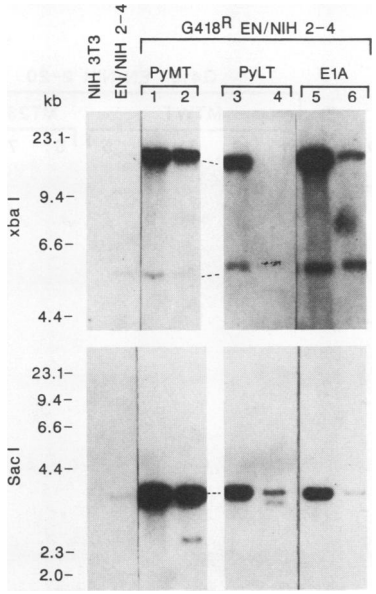

FIG. 2. Representative Southern blot analyses of *Xba*I and *Sac*I restriction enzyme digests of DNA from selected GRSs obtained from oncogene-transfected EN/NIH 2-4 cells placed into G418 selection. (See Materials and Methods for description of plasmids used in transfection.) Each numbered lane represents an independent GRS. Cellular DNAs from parental EN/NIH 2-4 and NIH 3T3 cells were used as positive and negative controls, respectively. All lanes were loaded with the complete restriction enzyme digest of 2.5 μg of cellular DNA. Hybridizations were carried out with a ^{32}P-oligolabeled *neo*-specific probe. Amplification and novel fragments are apparent compared with unrearranged parental EN/NIH 2-4 cellular DNA. (See Table 2 for summary of genomic rearrangements observed for all GRSs examined by Southern analysis.) Numbered dashes correspond to marker bands from the *Hin*dIII digest of lambda phage.

Table 1 summarizes the number of control- and oncogene-transfected EN/NIH 2-4 cell lines that were placed into G418 selection, the fraction of cell lines that yielded G418-resistant colonies, and the median and range of frequencies of GRSs observed. Since we have never observed GRSs for any parental or control-transfected EN/NIH 2-4 cell line placed into G418 selection, the inferred frequency of spontaneously arising GRSs for these cells is less than 10^{-8} per cell. In contrast, a number of Py MT-, Py LT-, and E1A-transfected hygromycin-resistant EN/NIH 2-4 cell lines gave rise to GRSs with frequencies ranging from at least one to three orders of magnitude greater than background. Although the fraction of E1A-transfected EN/NIH 2-4 cell lines giving rise to GRSs is greater than that for Py MT, which is greater than that for Py LT, and the median frequency of GRSs observed for Py MT-transfected EN/NIH 2-4 cell lines is two and three times greater, respectively, than that for Py LT and E1A (Table 1), the significance of these differences cannot be assessed from these data. Northern analyses of all GRSs studied demonstrated a *neo* transcript, whereas the transcript was undetectable in parental control transfectants (data not shown).

To identify genomic rearrangements involving *neo*-containing cellular DNA in randomly selected, independent GRSs, Southern blot analyses employing a *neo* probe were performed with a series of restriction enzymes for which recognition sites are absent (*Xba*I) or present once (*Bam*HI) or twice (*Sac*I) in the unrearranged parental retrovector (Fig. 1). Representative Southern blot results are shown in Fig. 2 (for *Xba*I and *Sac*I digests only). Compared with the unrearranged banding pattern of DNA digests from parental EN/NIH 2-4 cells, several types of genomic rearrangements involving *neo*-containing cellular sequences were observed in a number of DNA samples obtained from GRSs derived from oncogene-transfected EN/NIH 2-4 cells: high-level amplification of *neo*-containing cellular fragments (Fig. 2, lanes 1 to 3 and 5); appearance of a novel *neo*-containing cellular fragment (Fig. 2, lanes 2 and 4); and both amplification and appearance of a novel *neo*-containing fragment (Fig. 2, lane 2). Some samples demonstrated no apparent rearrangement of *neo*-containing cellular sequences (Fig. 2, lane 6).

The results of Southern analyses are summarized in Table 2 for all independent GRSs derived from oncogene-transfected EN/NIH 2-4 cells that were selected and expanded for examination. Whereas amplification of *neo*-containing cellular DNA was commonly observed (most commonly in GRSs derived from EN/NIH 2-4 cells transfected with Py MT compared with those transfected with Py LT and E1A), novel *neo*-containing cellular fragments were infrequently seen. When the latter were present, they were most often observed in conjunction with amplification. Surprisingly, only one (the 16-kbp *Xba*I fragment) of the two EN/NIH 2-4 parental *neo*-containing cellular fragments appeared to be involved by these genomic events.

Activation of *neo* by wild-type and mutant Py MT oncogenes in EN/NIH 2-20 cells. To substantiate the observations made with the wild-type oncogene and the EN/NIH 2-4 cell line, we repeated the above experiments with the clonally distinct EN/NIH 2-20 cell line and several related mutant oncogenes whose protein products differ in their associated biological and biochemical activities. We reasoned that if genomic rearrangements were truly related to one or more effects of the wild-type oncogene, then mutant oncogenes might demonstrate a mitigated or abrogated capacity to induce these genomic events. To this end, we compared the effects of wild-type and three mutant Py MT oncogenes (MT23, MT1015, and MT59b). We chose these mutant oncogenes for several reasons. First, constructs expressing these mutant oncogene products were readily available. Second, a large fraction of Py MT-transfected EN/NIH 2-4 cells had given rise to GRSs, and a large number of these GRSs had demonstrated genomic rearrangement (in particular, amplification) involving a *neo*-containing cellular fragment. Third, the protein products of these mutant oncogenes have been well characterized with regard to their transformation and associated in vitro TK and PIK activities (31). MT1015Ag has intact associated TK and PIK activities; MT23Ag has intact associated TK but deficient associated PIK activities; and MT59bAg is defective in both associated TK and PIK activities (31). Whereas cells transfected with MTWT are fully transformed, as defined by growth in soft agar and the capacity to form foci on a cell monolayer, cells transfected with the mutant MT oncogenes have mitigated or abrogated transformation properties which correlate with the activity of MTAg-associated PIK regardless of intact associated TK activity. Hence, cells expressing the MT1015Ag, which exhibits the greatest associated PIK activity of the three mutant Py MTAgs, are capable of growth in soft agar but have a diminished capacity to form foci on cell monolayers; cells expressing the MT23Ag, which has slight associated PIK activity, are incapable of growth in soft agar but exhibit slight activity in a focus formation assay; and cells express-

ing the MT59bAg, which has no associated in vitro PIK activity, demonstrate no properties of transformation (31).

As detailed in Materials and Methods, randomly selected, independent control- and oncogene-transfected hygromycin-resistant EN/NIH 2-20 cell lines were obtained following transfection of EN/NIH 2-20 cells with pZipHph, pZip (MTWT)hph, pZip(MT23)hph, pZip(MT1015), and pZip (MT59b)hph. As expected (9, 23, 31), only subclones of EN/NIH 2-20 transfected with MTWT appeared morphologically transformed. Cellular genomic integration and expression of the transfected oncogene were demonstrated in parental oncogene-transfected EN/NIH 2-20 subclones by Southern and Northern analyses, respectively, using a ^{32}P-oligolabeled MT probe (data not shown). A single-copy integrant of the oncogene with a unique site of genomic integration for each cell line was present in each oncogene-transfected EN/NIH 2-20 subclone examined with the exception of one; this one subclone (C1-6), derived from transfection of EN/NIH 2-20 cells with pZip(MTWT)hph, demonstrated amplification of the MTWT-containing locus.

Table 1 summarizes the number of control- and oncogene-transfected EN/NIH 2-20 cell lines that were placed into G418 selection, the fraction of cell lines that yielded G418-resistant colonies, and the median and range of frequencies of GRSs observed. Since we have never observed GRSs for any parental or control-transfected EN/NIH 2-20 cell line placed into G418 selection, the inferred frequency of spontaneously arising GRSs for these cells is less than 10^{-8} per cell. In contrast, 7 of 15 hygromycin-resistant EN/NIH 2-20 cell lines transfected with pZip(MTWT)hph gave rise to GRSs, a fraction similar to that observed for the EN/NIH 2-4 cell line transfected with pPyMT but distinctly larger than the fractions observed for EN/NIH 2-20 subclones transfected with the mutant Py MT genes. Of note, no GRSs were observed for the one wild-type MT-transfected EN/NIH 2-20 cell line (C1-6) that had demonstrated, by Southern analysis, amplification of the MTWT-containing cellular locus. Overall, the frequencies of GRSs observed per cell placed into G418 selection were one to two orders of magnitude less than those observed for the EN/NIH 2-4 cell line (Table 2). Northern analyses of all GRSs studied demonstrated a *neo* transcript, whereas the transcript was undetectable in parental control transfectants (data not shown).

To assess genomic rearrangement involving *neo*-containing cellular DNA in randomly selected, independent GRSs derived from wild-type and mutant Py MT-transfected EN/NIH 2-20 cells, Southern blot analyses employing a *neo* probe were performed with *Eco*RI (whose recognition site appears once in the unrearranged parental retrovector; see Fig. 1) in addition to the restriction enzymes (*Xba*I, *Sac*I, and *Bam*HI) employed in the analysis of DNA samples obtained from GRSs derived from oncogene-transfected EN/NIH 2-4 cell lines (see above). Representative Southern blot results (*Xba*I, *Sac*I, and *Eco*RI) are shown in Fig. 3. Compared with the unrearranged banding pattern of DNA digests from parental EN/NIH 2-20 cells, three types of genomic rearrangement involving *neo*-containing cellular sequences were observed in DNA samples obtained from GRSs derived from oncogene-transfected EN/NIH 2-20 cells: low-level amplification of *neo*-containing cellular fragments (Fig. 3, lanes 1 to 3, 6, and 7); appearance of a novel *neo*-containing cellular fragment (Fig. 3, lanes 1 to 8); and both amplification and appearance of a novel *neo*-containing fragment (Fig. 3, lanes 1 to 3, 6, and 7).

The results of Southern analyses are summarized in Table 2 for all independent GRSs derived from oncogene-trans-

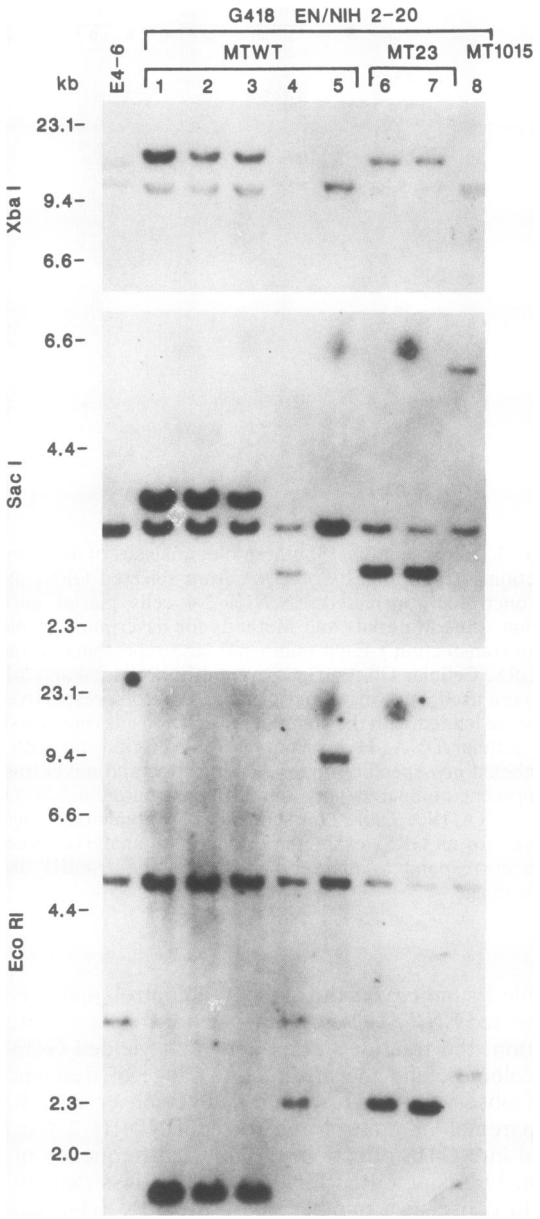

FIG. 3. Southern blot analyses of *Xba*I, *Sac*I, and *Eco*RI restriction enzyme digests of DNA from selected GRSs obtained from oncogene-transfected EN/NIH 2-20 cells placed into G418 selection. (See Materials and Methods for description of plasmids used in transfection.) Each numbered lane represents an independent GRS. E4-6 is a hygromycin-resistant subclone derived from transfection of EN/NIH 2-20 cells with pZip(MT1015)hph and is the parent cell line of the GRS represented in lane 8. Cellular DNAs from parental E4-6 and NIH 3T3 cells were used as positive and negative controls (not shown), respectively. All lanes were loaded with the complete restriction enzyme digest of 5 μg of cellular DNA. Hybridizations were carried out with a ^{32}P-oligolabeled *neo*-specific probe. Amplification and novel fragments are apparent compared with unrearranged parental E4-6 and EN/NIH 2-20 (not shown) cellular DNA. (See Table 2 for summary of genomic rearrangements observed for all GRSs examined by Southern analysis.) Numbered dashes correspond to marker bands from the *Hin*dIII digest of lambda phage.

fected EN/NIH 2-20 cells that were selected and expanded for examination. In comparison to the findings made on Southern analysis of DNA samples obtained from GRSs derived from oncogene-transfected EN/NIH 2-4 cells, several observations are noteworthy. First, whereas only a minority of GRSs derived from oncogene-transfected EN/NIH 2-4 cells demonstrated the appearance of a novel *neo*-containing cellular fragment, all DNA samples obtained from GRSs derived from Py MT-transfected EN/NIH 2-20 cells demonstrated the appearance of such a fragment (Fig. 3, lanes 1 to 8). In addition, when present, amplification was low level rather than high (Fig. 3, lanes 1 to 3, 6, and 7; compare with Fig. 2, lanes 1 to 3 and 5). Second, as was unexpectedly observed with the oncogene-transfected EN/NIH 2-4 cell lines, only one (the 14-kbp *Xba*I fragment) of the two EN/NIH 2-20 parental *neo*-containing cellular fragments appeared to be involved in these genomic events. Third, novel *neo*-containing cellular fragments of similar if not identical size were detected in several completely unrelated and independently derived GRSs (Fig. 3, lanes 1 to 3, 4, 6, and 7). In each instance where this was apparent, the genomic rearrangement appeared to include retrovector sequence(s), since analysis with *Sac*I (a restriction enzyme whose recognition site is present twice in the parental retrovector) revealed a novel *neo*-containing fragment that was either larger (Fig. 3, lanes 1 to 3) or smaller (Fig. 3, lanes 4, 6, and 7) than the unrearranged parental *neo*-containing fragment. In contrast, one GRS, the MTWT-transfected GRS (C4-1G-1a) represented in Fig. 3, lane 5, demonstrated *neo*-containing *Sac*I and *Bam*HI (not shown) fragments which were unchanged and *neo*-containing *Xba*I and *Eco*RI fragments which were decreased and increased in size, respectively, compared with the parental fragment from which the rearranged fragment was derived. These observations suggest that rearrangement in the C4-1G-1a GRS occurred in 5′-flanking cellular sequence upstream of the retrovector.

Relationship of *neo* activation to cell doubling time and level of oncogene expression. Cell doubling times and levels of oncogene expression were ascertained to assess whether these factors correlated with the capacity of a given oncogene-transfected EN/NIH cell line to activate *neo*. Doubling times for control- and oncogene-transfected EN/NIH cell lines were obtained from the linear regression of the $\log_{10}$ of cell counts (performed by trypan blue exclusion on triplicate samples sequentially harvested at four 24-h intervals following plating) versus time and ranged from 19 to 27 h (R^2 = 0.922 to 0.978) regardless of transformed phenotype (data not shown). Northern analyses of oncogene-transfected EN/NIH subclones demonstrated variable levels of oncogene expression (and equivalent levels of actin gene expression) between individual subclones (data not shown). There was no apparent correlation between the development of GRSs by a given oncogene-transfected EN/NIH subclone and its doubling time or level of oncogene expression (data not shown).

DISCUSSION

Using the cell system described in this and previous work (43), we have demonstrated that expression of specific DNA virus oncogenes may result in an increased rate of genomic rearrangements that are associated with activation of a transcriptionally silent, dominantly selectable reporter gene (*neo*). The types of rearrangement appear to be related to both the specific viral oncogene and the parent cell line used in transfection (Table 2). Although these observations seem to correlate with cell transformation based on the markedly fewer to no GRSs obtained with mutant Py MT oncogenes with attenuated transforming activity, they are not solely dependent on cell transformation, since Py LT, which does not transform established cells, is capable of inducing these genomic events. Furthermore, the development of *neo* activation does not correlate with cell generation time, at least for the proliferation rates observed in these experiments (data not shown).

Whereas the fraction of Py MT-transfected EN/NIH subclones giving rise to GRSs is similar for both the 2-4 and 2-20 cell lines, the frequencies with which DNA rearrangements are observed vary with the parent cell line used in transfection, since *neo* activation by Py MT is significantly less frequent in EN/NIH 2-20 than in 2-4 cells. Moreover, only one of the two parental *neo*-containing cellular fragments within a given EN/NIH subclone appears to be involved in genomic rearrangement. Taken together, these observations suggest that the frequency and specific characteristics of the rearrangements are dependent not only on the particular viral oncogene, but also on the site of integration within the cellular genome of the transcriptionally silent, dominantly selectable reporter gene (*neo*).

Since a myriad of DNA structural motifs may influence and potentiate amplification and recombination events (5, 11–13, 20, 22, 24, 36, 38, 41, 42, 47–50, 54–56), the difference in the capacity of each unique *neo*-containing locus in the EN/NIH 2-4 and 2-20 cell lines to engage in genomic rearrangement is not surprising. However, given the limitations of Southern analysis in detecting rearrangements, we cannot exclude the possibility that the second *neo* locus is involved in these genomic events. These analyses do not identify which *neo* locus is active in the G418-resistant EN/NIH 2-4 subclones that do not demonstrate genomic rearrangement of either *neo* locus by Southern analysis. In GRSs that do demonstrate genomic rearrangement of a *neo* locus, *neo* activation presumably occurs as the result of amplification (as in the majority of GRSs derived from oncogene-transfected EN/NIH 2-4 cells) or as the result of juxtaposition of *neo* with cellular enhancer elements formerly noncontiguous with *neo* (18, 19, 21) (as in at least some of the GRSs obtained from Py MT-transfected EN/NIH 2-20 cells). Novel *neo*-containing fragments occurring predominantly in the presence of high-level *neo* amplification (as in a minority of GRSs derived from oncogene-transfected EN/NIH 2-4 cells) presumably arise secondarily as the result of unequal crossover events between homologous amplified *neo* loci on sister chromatids (39) and are not primarily responsible for *neo* activation. Identification of genomic rearrangement involving one of the two *neo*-containing loci does not confirm that the *neo* locus associated with the genomic rearrangement is the locus from which *neo* is expressed, although transfection of rearranged *neo*-containing loci isolated from GRSs obtained in previous studies (43) and our preliminary (unpublished) observations with the rearranged *neo*-containing locus isolated from the C4-1G-1a GRS described in this work support this association.

The finding of novel *neo*-containing cellular fragments of similar if not identical size in completely unrelated and independently derived GRSs (Fig. 3) underscores further the role of site-specific genetic features in the development of genomic rearrangements. For example, novel fragments of similar size might arise if genomic sequences associated with hot spots of recombination were present near the *neo* locus. Given the surprising absence of rearrangements involving

the second *neo* locus present in each EN/NIH subclone and the striking difference in the types of genomic rearrangements associated with *neo* activation in oncogene-transfected EN/NIH 2-4 compared with 2-20 cells, it is unlikely that a genetic element present in the *neo*-containing retrovector itself predisposes to the development of similar genomic rearrangements. Furthermore, in contrast to the potential role of SV40 *ori* sequences in the development of GRSs derived from EN/NIH 2-4 cells exposed to a temperature-sensitive mutant of the SV40 LT antigen or TPA reported in previous work (43), we are unaware of any direct or indirect associations between *neo*-containing retrovector sequences and E1A or the Py MT and LT antigens used in the present investigations. To explore the role of the genetic locus in the development of genomic rearrangements, future studies must ascertain and characterize the sites of genomic integration of the *neo*-containing retrovector in the parent EN/NIH cell lines.

The mechanisms by which specific oncogenes result in the development of genomic rearrangements which activate the transcriptionally silent *neo* locus are not entirely elucidated by these studies. However, insertional activation of *neo* by transfected plasmid sequences is unlikely, given that GRSs are not observed in EN/NIH subclones transfected with control plasmids which contain LTRs or other enhancer elements but do not contain an oncogene. That *neo* activation and genomic rearrangement are related to introduction of a specific oncogene is underscored by the observations with the wild-type and mutant MT constructs: mutant MT oncogenes whose protein products have partially (MT1015 and MT23) or completely (MT59b) abrogated associated biological activities give rise to markedly fewer or no subclones that yield GRSs than the wild-type oncogene (MTWT). Although the fraction of wild-type MT-transfected subclones giving rise to GRSs may be sufficiently different from the fractions observed for the three mutant MT-transfected cell lines (Table 2), the differences among the latter are not sufficient to warrant firm conclusions about the potential relationship between specific biochemical activities associated with Py MT and the capacity of MT to induce genomic rearrangements. In interpreting these differences, particularly the absence of GRSs from EN/NIH cells transfected with MT59b, it should be emphasized that no GRSs have ever emerged from any parental or mock-transfected EN/NIH cell line that has been placed into G418 selection. Thus, *neo* activation occurs as a result of an effect related to the oncoprotein rather than to an enhancer element present in the construct used in transfection. However, specific functional associations between properties of the Py MT oncoprotein and induction of DNA rearrangements cannot yet be made.

This effect is not likely *trans* activation of *neo*, although this may be one mechanism by which *neo* is expressed in those oncogene-transfected EN/NIH 2-4-derived GRSs that do not demonstrate genomic rearrangement of the *neo* locus (Fig. 2, lane 6, and Table 2). Whereas E1A enhances or represses the expression of a number of cellular genes by affecting the activity of cellular transcription factors (2, 35), neither Py MTAg nor LTAg has been shown to behave as a direct or indirect cellular *trans* activator (3, 14, 25, 58). Furthermore, if *trans* activation were sufficient to activate the *neo* locus, then all cell lines expressing the oncoprotein would be expected to express *neo* and be resistant to G418. Since this is not observed, and since the development of *neo* activation does not correlate with the level of oncogene expression (data not shown), *trans* activation as a mechanism for *neo* activation and the development of genomic rearrangements is probably insufficient alone or is one in a series of events necessary for sufficient activation of *neo* at the concentrations of G418 used in these experiments.

Since *neo* activation is not ensured by the presence of the oncogene, subsequent events or the recruitment of additional factors is implied. These processes presumably arise stochastically. Identifying additional cellular factors leading to an increased capacity of a cell to activate a silent gene locus will be of interest for future investigations. Furthermore, it will be important to ascertain whether this capacity affects other dominantly selectable genomic loci that have been well studied with regard to their involvement in genomic rearrangements (e.g., the dihydrofolate reductase gene or the CAD [carbamyl-P synthetase, aspartate transcarbamylase, dihydroorotase] locus) and, if so, to identify the presence or absence of chromosomal intermediates that may be common to the development of these genomic rearrangements (e.g., dicentrics or double minutes associated with amplification [44]).

In summary, these studies demonstrate that specific DNA virus oncogenes induce genetic rearrangements in the EN/NIH model system independent of cell proliferation rate. If these observations reflect circumstances that occur in the course of neoplastic transformation, the cascade of events initiated by these oncogenes could regionally or generally result in instability of the genome, thereby facilitating a stochastic process by which subsequent rearrangement events are more likely to occur and contributing to the full and evolving expression of the neoplastic phenotype. Furthermore, given the variety of biochemical and genomic effects associated with the activities of oncogenes and their protein products, the mechanisms by which specific oncogenes result in genomic rearrangement are likely to be diverse and complex. In future studies, mutant oncoproteins whose biochemical properties have been well characterized will be employed in order to obtain greater insight into the mechanisms by which specific oncoproteins contribute to the development of genomic instability.

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