Gene Activation by Induced DNA Rearrangements

Lowell E. Schnipper, Victor Chan, John Sedivy, Parmjit Jat, and Phillip A. Sharp

ABSTRACT

A murine cell line (EN/NIH) containing the retroviral vector ZIPNeoSV(x)1 that was modified by deletion of the enhancer elements in the viral long terminal repeats has been used as an assay system to detect induced DNA rearrangements that result in activation of a transcriptionally silent reporter gene (neomycin phosphotransferase, neo) encoded by the viral genome. The spontaneous frequency of G418 resistance is less than 10⁻⁶, whereas exposure to the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) or the combination of UV irradiation plus TPA resulted in the emergence of drug resistant cell lines at a frequency of 5 per 10⁶ and 67 per 10⁶ cells, respectively. In several of the cell lines that were analyzed a low level of amplification of one of the two parental retroviral integrants was observed, whereas in others no alteration in the region of the viral genome was detected.

To determine the effect of the SV40 large T antigen on induced DNA rearrangements, EN/NIH cells were transfected with a temperature sensitive (ts) mutant of SV40 T. Transfectants were maintained at the permissive temperature (33°C) for varying periods of time (1-5 days) in order to vary SV40 T antigen exposure, after which they were shifted to 39.5°C for selection in G418. The frequency of emergence of drug resistant cell clones increased with duration of exposure to large T antigen (9-52 per 10⁶ cells over 1-5 days, respectively), and all cell lines analyzed demonstrated DNA rearrangements in the region of the neo gene. A novel 18-kilobase pair XbaI fragment was cloned from one cell line which revealed the presence of a 2.0-kilobase pair EcoRI fragment containing an inverted duplication which hybridized to neo sequences. It is likely that the observed rearrangement was initiated by the specific binding of large T antigen to the SV40 origin of replication encoded within the viral genome.

The investigations with phorbol esters, UV light, and the SV40 large T antigen demonstrate the utility of the EN/NIH cell lines for the study of induced DNA rearrangements and support the future use of this system to investigate the mechanism by which varied stimuli or specific gene functions promote DNA rearrangements.

INTRODUCTION

The characteristics of a malignant cell population do not remain constant. Clones derived from the same tumor demonstrate heterogeneity in the expression of important biological properties such as propensity to develop metastasis, drug resistance, expression of hormone receptors, and cell surface antigen expression (1-3). Although epigenetic factors may play a role, the primary basis of the heterogeneity is the genetic instability associated with the neoplastic phenotype (4). Many forms of human cancer have nonrandom karyotypic abnormalities. Some result in activation of cellular oncogenes (e.g., t8;14 in Burkitt's lymphoma and t9;22 in chronic myelogenous leukemia) (5-8), while others are believed to cause loss of tumor suppressor genes (13q—deletion in retinoblastoma). Nonrandom karyotypic abnormalities are also associated with tumor progression. B-cell lymphomas which evolve from indolent to more aggressive patterns of growth demonstrate nonrandom changes such as 6q—deletion or trisomy 7 or 12 (9), and as chronic myelogenous leukemia evolves to the blast crisis stage multiple chromosomal abnormalities are observed (2). These macroscopic changes may be representative of a wider array of DNA rearrangements that occur at a microscopic level. In support of this is the demonstration of changes in restriction fragment length polymorphisms at certain loci in a high percentage of malignant melanomas studied (10).

Gene amplification, another manifestation of genetic instability, has been most closely associated with the immortalized or neoplastic phenotypes and has been correlated with increasing biological aggressiveness of certain human cancers (11) and the expression of drug resistance (12). Its biological basis is not known, but evidence has recently been presented in rodent cells for the expression of an "amplificator phenotype," characterized by an accelerated rate of gene amplification (13).

MATERIALS AND METHODS

Cell Lines, Plasmids, and Culture Conditions. EN/NIH cell lines were a generous gift of Dr. Richard Mulligan. The cell lines were established by infection of NIH 3T3 cells with a lysate from the 2 packaging cell line (14) that was transfected previously with a variant of the retroviral vector ZIPNeoSV(x)1 (15) that contains a 100-base pair XbaI-PvuII deletion encompassing the transcriptional enhancer in the 3'LTR (plasmid pZIPNeoSV enh) (Fig. 1a). Infected cells were subjected to single cell cloning in the absence of G418 selection pressure. Colonies demonstrating integrated retroviral DNA as determined by Southern blot hybridization analysis, and the absence of retroviral transcripts by Northern analysis, were selected for further study. EN/NIH 2-4 and EN/NIH 2-20 are two such cell lines used in the present investigations; each contains two retroviral integrants.

The plasmid pLTRasAS8 (16) is a derivative of ZIPSV40 (17) except that it contains the tsAS8 mutation (18) in the large T antigen coding sequence, and a deletion of the XhoI-XhoI fragment that encodes neo, the SV40ori and the pBRori. The plasmid PY3 carries the gene for hygromycin resistance (19).

Cells were propagated in DME supplemented with 10% calf serum, at 37°C, in an atmosphere of 5% CO₂, unless otherwise stated. Selection in G418 was performed using 300 µg/ml of the active drug; and in hygromycin using 150 µg/ml (w/v total drug). In studies with the tumor promoting agent, TPA (Sigma Chemical Co., St. Louis, MO), cells were propagated in DME containing 10 ng/ml of TPA for 3 days, after which they were subcultured into DME containing G418 plus 10 ng/ml of TPA. The TPA was removed after 4 days, and the G418 selection continued for 21 days. When UV irradiation plus TPA was used, the cells were exposed to 5 J/m², subcultured into DME containing 10 ng/ml TPA for 72 h, and then exposed to G418 selection. Following exposure to UV alone, cells were either incubated in DME for 24 h prior to selection or immediately placed in G418. Transfections were performed by the calcium phosphate coprecipitation technique of Graham and Vandenberg (20), as modified by Parker and Stark (21), and included exposure to 100 µg/ml chloroquine for 4 h immediately following transfection (22). Forty-eight h after transfection the cells were placed in selective medium.

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The abbreviations used are: LTR, long terminal repeat; DME, Dulbecco's minimal essential medium; TPA, 12-O-tetradecanoylphorbol-13-acetate; kb, kilobase pairs.
5 R. DeHerrn and R. Mulligan, personal communication.
RESULTS

Spontaneous Frequency of Resistance to G418. The frequency of spontaneous G418 resistance in EN/NIH 2-4 and EN/NIH 2-20 cells was less than 1 per $5 \times 10^5$ and $3 \times 10^7$ plated cells, respectively, when scored as colonies emerging after selection in G418. In fact, no drug resistant colonies were observed after 14 days with either cell line. Since the background level of spontaneous G418 resistance in EN/NIH 2-4 and EN/NIH 2-20 cells was less than 1 per $5 \times 10^5$ and $3 \times 10^7$ plated cells, respectively, it was anticipated that the interaction between SV40 large T antigen and the SV40ori contained within the integrated en genome plasticity has not been adequately examined. The utility of the EN/NIH cell lines for this purpose was examined using the restriction enzymes BamH1, XbaI, and EcoRI and neo probe. The absence of the same parental fragment and the presence of identical novel DNA fragments in which the neo signal was 3-5-fold amplified were observed in each cell line analyzed, one of which is demonstrated in Fig. 2a.

In a separate experiment, exposure of EN/NIH 2-4 cells to 10 ng/ml of TPA resulted in the appearance of G418 resistant clones at a frequency of 5 per $10^6$ cells. However, these cells exhibited diminishing levels of drug resistance during passage as judged by progressively slower growth in G418. By reducing the concentration of G418 to 150 µg/ml active drug, the clones exhibited a stable degree of G418 resistance. Hybridization analysis failed to reveal the presence of a novel DNA fragment in any cell line studied, although of 7 clones that were analyzed, 2 demonstrated approximately 3-fold amplification of one of the retroviral integrants, one of which is shown in Fig. 2b, e.g., EN/NIH 2-4 TPA3.

UV irradiation (254 nm) at a 50% lethal dose did not result in the appearance of G418' clones under conditions in which 10' surviving cells were studied. However, UV irradiation of EN/NIH 2-4 cells followed by exposure to 10 ng/ml TPA for 3 days prior to selection resulted in the appearance of a high frequency of independent clones (67 G418 resistant clones per $10^6$ cells) exhibiting a stable drug resistance phenotype. Thus, it appears that UV treatment, although negative by itself, was capable of significantly enhancing the effect of TPA (Table 1).

One of 5 analyzed cell lines demonstrated approximately 3-fold amplification of 1 of the 2 parental retroviral fragments but no novel fragments were observed [e.g., EN/NIH 2-4 UVT2 (Fig. 2b)]. No differences from the parental fragments were observed in the remaining 4 UV/TPA clones studied, one example of which, EN/NIH 2-4 UVT1, is shown in Fig. 2b.

Effect of SV40 Large T Antigen on the Frequency of Resistance to G418. The potential effect of various oncogenes on genome plasticity has not been adequately examined. The utility of the EN/NIH cell lines for this purpose was examined using the SV40 large T antigen gene. This gene was selected because it was anticipated that the interaction between SV40 large T antigen and the SV40ori contained within the integrated enhancer minus retrovectors might result in an abortive replication event, which would be recombinogenic. To allow precise regulation of the time of exposure to functional large T antigen, a tight temperature sensitive, replication defective mutant was used (tsA58).

The plasmid pLTRtsA58 was stably introduced into the EN/NIH 2-4 cell line by cotransfection with the kph gene (plasmid pY3) to establish the cell lines termed EN/NIH tsA/Y. All manipulations were at the nonpermissive temperature (39.5°C). Hygromycin resistant clonal cell lines were tested for expression of large T antigen by in situ indirect immunofluorescence using a hamster anti-large T polyclonal antibody. Approximately 95% of resistant cell lines demonstrated strong and uniform nuclear staining at 33°C (the permissive temperature) but not at 39.5°C. These observations suggest that large T expression could be modulated by the appropriate temperature shifts (also see (16) for description of tsA58 regulation). To determine the effect of large T antigen exposure on the frequency of emergence of G418 resistant variants, one such EN/NIH tsA/Y cell line was shifted to 33°C and maintained at this temperature for 1-5 days, at which time the cells were shifted back to the nonper-
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Table 1 Induction of G418 resistance in EN/NIH cell lines

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>G418 resistant clones/10&lt;sup&gt;6&lt;/sup&gt;</th>
<th>Parent cells</th>
<th>Genomic alteration&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>EN/NIH 2-4 or 2-20</td>
<td>Novel amplified DNA fragment, loss of one parental retroviral fragment</td>
</tr>
<tr>
<td>TPA (10 ng/ml)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5</td>
<td>EN/NIH 2-20</td>
<td>One of 2 parental retroviral amplified fragments</td>
</tr>
<tr>
<td>UV/TPA (LD&lt;sub&gt;50&lt;/sub&gt;)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0</td>
<td>EN/NIH 2-4</td>
<td>N/A</td>
</tr>
<tr>
<td>UV/TPA (10 ng/ml)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>67</td>
<td>EN/NIH 2-4</td>
<td>Amplification of one of the parental retroviral DNA fragments</td>
</tr>
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</table>

<sup>a</sup> To establish the frequency of G418 resistance following exposure of either EN/NIH 2-4 or 2-20 cells to a specific stimulus, treated cells were placed under selection pressure in 600 μg/ml (300 μg/ml of active drug). G418 and drug resistant clones were quantitated on day 14.

<sup>b</sup> DNA from G418 clones were digested with appropriate restriction endonucleases and analyzed by Southern hybridization as described in “Materials and Methods.”

<sup>c</sup> Cells were exposed to TPA from 3 days prior to, through 4 days following initiation of G418 selection. These clones were derived from a single plate and therefore may represent siblings.

<sup>d</sup> EN/NIH cells were exposed to a 50% lethal dose (LD<sub>50</sub>) of UV<sub>ir</sub> irradiation which was followed by TPA (10 ng/ml) for 72 h, before addition of G418.

missive temperature and placed under selection in G418. A positive linear relationship between duration of exposure to SV40 large T antigen and the emergence of G418 resistant colonies was observed [9–50 colonies per 10<sup>6</sup> cells (Table 2)]. No G418 resistant colonies were observed in nontransfected EN/NIH 2-4 cells maintained at 33°C. In contrast, a single G418 resistant colony was observed in an EN/NIH tsA/Y control population maintained at 39.5°C. When subjected to Southern blot hybridization analysis, nonamplified, novel DNA fragments that hybridized to the neo probe were identified in all 4 cell lines examined. A cell line selected for further study, labeled EN/NIH tsA/Y/1d4, since it emerged after 1 day at 33°C and was the fourth clone picked, demonstrated novel 2.0-kbp EcoRI and 18-kbp Xbal fragments (Fig. 3).

Since Xbal does not cleave the retroviral vector, the novel Xbal fragment was isolated by recombinant methods to define the rearrangement in greater detail. The initial retroviral loci were isolated from the parental EN/NIH 2-4 cell line by circularization of Xbal digested genomic DNA, direct transformation into E. coli, and selection on kanamycin plates. This was possible since the ZIPNeoSV(x)<sup>s</sup> vector backbone contains the bacterial pBR322 plasmid origin of replication. The novel Xbal fragment from EN/NIH tsA/Y/1d4 was cloned into a cosmid vector [the charomid, 9–28 (24)]. A physical map of the rearranged fragment was derived by analysis of restriction endonuclease digestion products. The unique 2.0-kbp EcoRI fragment which hybridized to the neo probe was contained within a 3.75-kbp SalI fragment present as an inverted repeat. This latter fragment was contained within a 5.8-kbp SalI segment that spans both of the retroviralLTRs (Fig. 1b).

To confirm that the cloned DNA fragment was associated with activation of the neo gene, NIH 3T3 cells were transfected with either the rearranged Xbal fragment, termed tsA/Y/1d4, the parental Xbal fragment, 2-4Xba/3-1, or the charomid vector 9-28. The rearranged fragment generated G418 resistant clones at the frequency of 5 x 10<sup>−3</sup>, whereas the others failed to transfer the G418 resistant phenotype. The 5.8-kbp SalI fragment which contains the inverted duplication was subcloned into a plasmid vector (bluescript). This smaller fragment transferred the G418 resistant phenotype with similar efficiency to NIH cells. Three other subclones derived from the 18kbp Xbal fragment were negative in the same assay.

DISCUSSION

A murine cell line (EN/NIH) containing the retroviral vector pZIPNeoSV(x)<sup>s</sup> that has been modified by the deletion of its transcriptional enhancers has been used to detect induced DNA rearrangements. This constitutes an assay for stimuli that induce DNA rearrangements since these activate a reporter gene encoded, but not expressed, by the viral genome.

Each of the EN/NIH cell lines used in these studies contains 2 integrated copies of the retrovector and is sensitive to G418. The spontaneous frequency of events generating G418 resistance was extremely low, since no resistant clones were observed in the absence of a specific stimulus. This is consistent with findings of Thomas et al. (25), who found that the spontaneous frequency of G418 resistance in mouse cells was <10<sup>−7</sup>. The insignificant background suggests that the EN/NIH system is sufficiently sensitive to detect the emergence of G418 resistance at a frequency greater than 5 x 10<sup>−7</sup>. The anticipated mechanisms by which neo activation might occur include amplification of the neo gene which under basal conditions is not transcribed at a detectable level (data not shown), a rearrangement that positions an enhancer sequence in sufficiently close proximity to neo to stimulate transcription, a mutation resulting in overproduction of a transcription activating factor, e.g., AP-1, which binds to a recognition sequence in the retroviral integrant, and hypomethylation of regulatory sequences in flanking cellular DNA.

Exposure to the tumor promoting agent TPA resulted in the appearance of G418 resistant colonies having two distinct genotypes. In one group, cell clones were observed at a frequency of 5 per 10<sup>6</sup> that demonstrated low level, unstable drug resistance. No structural alterations in the region of the retroviral genome were observed by Southern analysis although in several cell lines a low level of amplification of the neo sequences was observed. The other, arising at a frequency of 5 x 10<sup>6</sup> cells, was the emergence of stably resistant clones, in conjunction with the appearance of an amplified, novel DNA fragment and loss of one parental fragment.

No G418 resistant clones emerged after exposure to a 50% lethal dose of UV irradiation, but the combination of UV light and TPA proved to be effective at generating drug resistance at an approximately 10-fold higher frequency than TPA alone (67 per 10<sup>6</sup> cells). Examination of these clones by Southern blot hybridization analysis revealed a low level of neo amplification in one cell line while no novel fragments or gene amplification events were detected in the others.

Gene amplification has been described previously in association with the activation of a functionally silent gene. When rodent cells containing a promotorless thymidine kinase (TK) gene were placed under selection pressure, clones expressing the TK(+) phenotype were associated with 20–30-fold amplification of the parental TK gene, without the appearance of novel DNA fragments (27). This type of activation event requires a low basal transcription level from the functionally silent gene, so that a simple gene dosage increase can cause “activation” in a phenotypic sense. Since such an event was never observed during multiple attempts at eliciting spontaneous emergence of G418 resistance, the expression level of the neo gene in the enhancer deleted LTR construct must be vanishingly low (Northern blot hybridization consistently failed to produce any signal, data not shown). The relationship between
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Fig. 2. Southern blot hybridization analysis of G418 resistant cell lines which emerged after exposure to TPA and UV. Total DNA was extracted from parental or G418 resistant cells and subjected to Southern blot hybridization analysis using a neo probe (see "Materials and Methods"). UVT1 and UVT2 are cell lines that arose after exposure of the EN/NIH 2-4 cell line to UV plus TPA (Table 1, Line 5). TPA 3 is a cell line that arose after exposure of the EN/NIH 2-4 cell line to TPA alone (Table 1, Line 2). The restriction enzymes used in the analysis are indicated below each lane, a: lanes 1, 3, 5, EN/NIH 2-4; lanes 2, 4, 6, EN/NIH 2-4 IL. b: lanes 1, 4, 8, EN/NIH 2-4; lanes 2, 5, 9, UVT 1; lanes 3, 6, 10, UVT 2; lanes 7, 11, TPA 3. *, fragments demonstrating either amplification or rearrangement.

Table 2 Exposure to SV40 large T antigen and emergence of G418 resistant cells in EN/NIH cell lines

<table>
<thead>
<tr>
<th>Exposure (days)</th>
<th>G418/10^6 cells</th>
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<tbody>
<tr>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>45</td>
</tr>
<tr>
<td>5</td>
<td>52</td>
</tr>
</tbody>
</table>

* EN/NIH cells stably transfected with the tsA gene of SV40 were shifted from 39.5°C to 33°C for the indicated time (days) after which they were shifted to 39.5°C in 300 μg/ml of active G418, for 14 days.

Fig. 3. Southern blot hybridization analysis of the G418 resistant clone tsA/Y/104 which emerged after exposure to SV40 large T antigen. The restriction enzymes used in the analysis are indicated below each lane. Lanes 1, 3, 5, 7, EN/NIH 2-4; Lanes 2, 4, 6, 8, tsA/Y/104. Southern blot analysis was performed as described in "Materials and Methods" and in the legend of Fig. 2.

exposure to TPA or UV light plus TPA, gene amplification, and neo activation is not obvious, and a detailed investigation of the mechanisms involved is beyond the scope of this study. The G418 resistant cells arising after exposure to TPA or UV plus TPA and demonstrating parental retroviral DNA conformations may have undergone a DNA rearrangement that is undetected since the restriction endonuclease analysis was limited to approximately 20–25 kbp of cellular DNA flanking the inserted neo gene. A more complete analysis would involve the evaluation of substantially larger fragments by digestion with restriction enzymes having an 8-base pair recognition sequence combined with pulse-field electrophoresis. Furthermore, the present studies have not examined the issue of DNA methylation and its possible influence on the expression of the neo gene.

The ZIPNeoSV(x)1 vector contains SV40 sequences [base pair coordinates 5171 through 161 of the standard SV40 map; Tooze (28)], which include the early mRNA start site and origin of replication, the 21-base pair repeats, and approximately two-thirds of the first 72-base pair repeat (29, 30). The factor JunB or AP-1 binds to a consensus octanucleotide sequence, C(G)TGACTC(A)A, contained within the remaining 72-base pair enhancer fragment. TPA has been demonstrated to increase the activity of the JunB factor. This suggests the possibility that TPA may facilitate generation of G418 resistant colonies by increasing levels of JunB activity to establish a level of sufficient transcriptional activity, so that gene amplification can produce adequate amounts of the neo protein. This mechanism may be particularly relevant to the clones that were weakly G418 resistant.

Rearrangements that activated the neo gene were detected in EN/NIH cells modified to express a temperature sensitive mutant of the SV40 large T antigen or A gene. SV40 large T
antigen exposure was directly controlled using temperature shifts and was correlated with the frequency at which G418 resistant clones appeared, increasing 9-fold from 1 to 5 days of exposure. Each clone analyzed demonstrated the presence of novel neo containing DNA fragments, none of which were amplified. Physical mapping of a cloned Xbal genomic fragment from one of these cell lines (EN/NH1tsA/Y1D4) revealed the presence of a 2.0-kbp EcoRI segment that is an inverted duplication, which contains the activated neo gene. Although speculative, it is plausible that such rearrangements can be initiated by the specific binding of large T antigen to the SV40 origin of replication. Murine cells are commonly considered nonpermissive for SV40 replication; however, a very low level of large T antigen stimulation of replication cannot be excluded by previous studies. The DNA conformations produced by these abortive priming events may be highly recombinogenic (31). The suggested interaction of SV40 large T antigen with the viral origin of replication may be analogous to the observations of Murnane (32) who studied spontaneous DNA re-arrangements involving an integrated plasmid that carried a promoter-less neo gene and the SV40ori, in SV40 transformed human fibroblasts. G418 resistance was observed in association with tandem duplications of the plasmid and flanking cellular DNA such that ori sequences having bidirectional transcriptional regulatory activity were now oriented 5’ to the neo gene. This rearrangement was presumed to result in transcriptional activation of the neo gene. Other investigators also documented the presence of tandem duplications at the site of SV40 inte- gration in viral transformants of rodent cells (33, 34). As a result of these structural alterations a cellular enhancer element may have been moved into sufficiently close proximity to the neo gene to augment its transcription. Indeed, preliminary observations demonstrate the presence of at least four trans- scripts that hybridize with neo, ranging in size from 2.0 to 8.5 kbp (data not shown).

These experiments, involving diverse stimuli such as phorbol esters, UV irradiation, and the SV40 large T antigen, demonstrate the utility of the EN/NH1 cell lines as a biological assay system for the study of gene activation occurring in association with DNA rearrangements. In this regard, the most important aspect of this system is that the background level of neo gene activation is very low. Furthermore, analysis of rearrangements is facilitated by the ability to easily rescue retroviral DNA and flanking cellular sequences for detailed structural and functional examination. This system should be useful for the determination of the activity of a variety of agents or specific gene functions in promoting DNA rearrangements and to undertake an analysis of the basis of genetic instability in neoplasia.

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