Retention and Expression of the Left End Subfragment of the Herpes Simplex Virus Type 2 BglII N DNA Fragment Do Not Correlate with Tumorigenic Conversion of NIH 3T3 Cells

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SUMMARY
Cotransfection experiments have been carried out using recombinant plasmids pAG60, conferring resistance to antibiotic G418, and pXho3 which contains the left end subfragment (map coordinates 0.583 to 0.596) of the transforming herpes simplex virus type 2 BglII N DNA fragment and encodes the 36K polypeptide associated with the viral ribonucleotide reductase activity. Several NIH 3T3 cell clones resistant to G418 and having morphological changes commonly observed for transformed NIH 3T3 cells were isolated and examined for the presence and stable retention of the viral sequences. Seven of the clones that retained the transfected viral sequences were analysed for the expression of the 36K polypeptide and the tumorigenic phenotype. The results gathered from these studies show that neither the retention of the viral DNA nor the expression of the 36K polypeptide correlated with tumorigenic conversion of these cells.

Several laboratories have demonstrated the oncogenic potential of herpes simplex virus type 2 (HSV-2) in rodent primary embryo fibroblasts and/or in NIH 3T3 cells (Duff & Rapp, 1977; Kessous et al., 1979; Kimura et al., 1975; Macnab, 1974). Two DNA fragments of the viral genome, BglII C and the more extensively studied BglII N, have been associated with the transforming activity (Reyes et al., 1979; Galloway & McDougall, 1981; Jariwalla et al., 1980, 1983, 1986; Hayashi et al., 1985). The BglII N fragment and a 737 bp subfragment thereof (BC24) were shown to induce the transformation of NIH 3T3 and rat embryo cells in a focus formation assay (Galloway et al., 1984). In these experiments the viral sequences, when present in the transformed cell lines, were detected in very low copy number and were lost upon passage in culture and/or tumour derivatives (Cameron et al., 1985; Galloway & McDougall, 1981; Galloway et al., 1984). These observations have led to the hit-and-run hypothesis, in which a region resembling an insertion sequence (IS) identified in the 737 bp subfragment of BglII N was proposed to act as a mutagen producing the first of a series of events that lead to the expression of the transformed phenotype (Galloway et al., 1984).

To test this hypothesis, Brandt et al. (1987) and Pilon et al. (1979) have carried out experiments designed to evaluate the mutagenic potential of the transforming left end of the BglII N fragment. The results obtained from both studies demonstrated no significant mutagenic effect, thus failing to support the hit-and-run hypothesis. The mapping of the ribonucleotide reductase 36K subunit gene to the left portion of the BglII N (Galloway et al., 1982; Preston et al., 1984; Suh et al., 1983) raised the possibility that transient or stable expression of 36K could participate in the induction of the transformed phenotype (Huszar & Bacchetti, 1983). To test the latter hypothesis, NIH 3T3 cells were cotransfected with the
neomycin (antibiotic G418) resistance (*neo*) and 36K polypeptide genes, respectively contained in the recombinant plasmids pAG60 (Colbère-Garapin *et al.*, 1981) and pXho3 (Suh *et al.*, 1983).

Several cell lines having resistance to G418 and the morphological changes commonly observed in transformed NIH 3T3 cells were selected and tested for the retention of the viral sequences. Among the clones that retained the pXho3 sequences, seven were randomly chosen and examined for the expression of the 36K protein and the tumorigenic phenotype. Our results suggest that neither the retention nor the expression of the *BglII* N left end sequences correlate with the tumorigenic conversion of the NIH 3T3 cells.

The seven clones (H2, H4, B01G, B03G, B09G, B11G, D1G) analysed in this study were isolated from three different cotransfection experiments using the recombinant plasmids pXho3 and pAG60, as previously described (Saavedra & Kessous-Elbaz, 1985). The pXho3 plasmid contains the left end 2.2 kb subfragment of *BglII* N, corresponding to nucleotides 157 to 2416 of the sequence established by Galloway & Swain (1984). It contains the complete sequence coding for the 36K subunit of the HSV-2 ribonucleotide reductase and the IS-like structure mentioned above (Fig. 1).

For all the clones the cellular morphology was fibroblastic with criss-crossing and moderate piling up. They were able to grow at high density in 2-5% calf serum whereas untransformed NIH 3T3 cells stayed flat and contact-inhibited at this serum concentration. They formed colonies in soft agar at frequencies ranging from 5 to 17.5%; however, the colonies were small compared to those obtained with ras-transformed NIH 3T3 cells. Normal and pAG60-transfected cells formed colonies with 2.3 to 2.7% efficiency. The tumorigenic potential of the clones and control NIH 3T3 cells was tested by subcutaneous injection of $1 \times 10^6$, $2 \times 10^6$ and $5 \times 10^6$ cells into nude mice. The latent period in this assay was limited to 15 weeks because we, as have others (Blair *et al.*, 1982), observed that tumours might occur 14 to 15 weeks after injection of high concentrations ($1 \times 10^7$) of normal NIH 3T3 cells. Under these conditions, H2, B01G, B09G and D1G cells formed tumours at all the inoculation doses tested in all the animals after latent periods of 8, 5, 5 and 4 weeks, respectively (Table 1). Tumour cell lines H2T, B01T, B09T and D1T were derived from tumour samples and expanded in selective medium. NIH 3T3 control cells as well as clones H4, B03G and B11G never formed palpable tumours within 15 weeks, when the assay was stopped, and were considered non-tumorigenic.

To investigate the retention of the 36K protein sequences, the DNAs of the selected clones and derived tumours were analysed by Southern blot hybridization (Saavedra & Kessous-Elbaz, 1985), using *BglII, XhoI* and *SacI*. The recombinant pXho3 has no restriction site for *BglII* (Fig. 1). *XhoI* and *SacI* endonucleases respectively excise the 2.2 kb ‘*Xho3*’ insert and a 1.5 kb fragment (Fig. 3a, lanes 1 and 3). Double cleavage of pXho3 with *XhoI* and *SacI* should result in four bands of 4.6 kb (pBR vector moiety), 1.5, 0.6 and 0.2 kb (‘*Xho3*’ moiety) (Fig. 3a, lane 3); however, the smallest fragment was never detected, probably because it ran out of the gel during electrophoresis. As this fragment is not contained in the sequences coding for the 36K protein, no special effort was made to detect it.

As shown in Fig. 2, hybridization of *BglII*-digested DNAs from the selected clones and the derived tumour cell lines with the ‘*Xho3*’ probe produced a hybridization pattern that was
Fig. 2. Southern blot analysis of DNAs from all the cell lines and the tumour derivatives. The DNAs (20 μg per lane) were cleaved with BgIII and probed with the ‘Xho3’ fragment. (a) DNAs from (lane 1) NIH 3T3 cells, (lane 2) H2 cells, (lane 3) H4 cells, (lane 4) B01G cells, (lane 5) B03G cells, (lane 6) B09G cells, (lane 7) B11G cells and (lane 8) D1G cells. (b) DNAs from tumour derivatives. (lane 1) H2T cells, (lane 2) B01T cells, (lane 3) B09T cells and (lane 4) D1T cells. Reconstruction lane REC contains 40 pg of pXho3 DNA digested with XhoI. Molecular sizes are indicated in kb.

Table 1. Correlation between 36K polypeptide expression and the tumorigenic phenotype

<table>
<thead>
<tr>
<th>Clone</th>
<th>Size of insert (kb)</th>
<th>Expression of 36K</th>
<th>Tumorigenicity</th>
<th>Latent period (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2</td>
<td>9.4</td>
<td>+</td>
<td>+</td>
<td>8</td>
</tr>
<tr>
<td>H4</td>
<td>6 to 25</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B1G</td>
<td>8 to 19</td>
<td>-</td>
<td>+</td>
<td>5</td>
</tr>
<tr>
<td>B3G</td>
<td>9 to 12</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B09G</td>
<td>14.5</td>
<td>-</td>
<td>+</td>
<td>5</td>
</tr>
<tr>
<td>B11G</td>
<td>8 to 12</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D1G</td>
<td>21</td>
<td>+</td>
<td>+</td>
<td>4</td>
</tr>
</tbody>
</table>

specific for each clone. H2, B09G and D1G cells showed unique bands of about 9.4, 14.5 and 21 kb respectively (Fig. 2a, lanes 2, 6, 8). Each clone contains approximately one copy of the ‘Xho3’ sequences as estimated from a reconstruction experiment (Fig. 2, REC). In the other cell lines, H4, B01G, B03G and B11G, the hybridization profiles exhibited multiple signals, indicating that several copies of the transfected sequences had been integrated in the host DNAs. H4 cells harboured approximately 12 copies of the ‘Xho3’ sequences distributed in BgIII-restricted fragments ranging from 6 to 25 kb (Fig. 2a, lane 3); in B01G DNA, the viral insertions were distributed over six fragments ranging from 8 to 19 kb (lane 4); in B03G we detected three major bands of 9, 10 and 12 kb (lane 5); lastly, in B11G cells the bands that showed the most intense signals were 8, 10 and 12 kb (lane 7).
The maintenance of the transfected sequences was examined in the four tumour-derived cell lines. The hybridization profiles as well as the intensity of the signals appeared unchanged in all of them (Fig. 2b, lanes 1 to 4), except for B01T cells where the 12 kb band was missing and the hybridization signal was less intense (Fig. 2a, lane 4; b, lane 2). This might be due either to a decrease in copy number during tumour growth or to dilution of the tumour sample by contaminating normal tissues. After removal of the viral probe, the filters were re-probed with the neo probe. All the clones retained several copies of the transfected neo sequences (data not shown). In order to determine whether the restricted fragments spanning the 36K coding sequences were integrally retained, the DNAs of all the clones were triply digested with BgII, XhoI and SacI. Hybridization with 'Xho3' probe revealed the expected SacI 1-5 kb band in all clones (Fig. 3b). The XhoI/SacI 0-6 kb band which contains the 5' end sequences of the 36K gene was also detected in H4, B01G and B03G (Fig. 3b, lanes 2 to 4); thus, the 36K polypeptide coding sequences are apparently retained in these clones. In the remaining DNAs (H2, B09G, B11G and D1G) no signal was detected at the 0-6 kb position (Fig. 3b, lanes 1, 5, 6, 7). This might be due to a partial or complete loss of the 0-6 kb band but it could also result from inactivation of the 5' XhoI recognition site. Finally, all the tested clones exhibited additional bands of various molecular sizes (Fig. 3b) which could represent incomplete or rearranged copies of the 'Xho3' subfragment. The nature and extent of the rearrangements were not determined in this analysis.

Using the immunoblotting technique (Towbin et al., 1979), we then examined the same clones for the expression of subunit 2 of the HSV-2 ribonucleotide reductase; this 36K protein corresponds to the major product entirely encoded in the 'Xho3' subfragment (Bacchetti et al., 1986).
Fig. 4. Detection of the 36K polypeptide by immunoblotting using P9 antiserum. Proteins (100 µg for HSV-infected cells and 200 µg for controls and tested cells) were separated by SDS-PAGE and electroblotted to nitrocellulose filters. Protein extracts were purified from HSV-2-infected (lane 8) and mock-infected (lanes 2, 5) NIH 3T3 cells, D1G cells (lane 3), B11G cells (lane 6) and H2 cells (lane 7). The controls included are HSV-1-infected (lane 1) and HSV-2-infected (lane 3) BHK cells and HSV-2-transformed hamster cells (lane 4).

1984, 1986; Cohen et al., 1985; Galloway et al., 1982; Jenkins & Howett, 1984; McLauchlan & Clements, 1983; Swain & Galloway, 1986). The immunoblots were probed with two different polyclonal antibodies (P9 and RS21) at a dilution of 1/500. P9 antiserum was raised against a synthetic peptide corresponding to the nine carboxy-terminal amino acids of the 36K protein (Cohen et al., 1986) and RS21 was raised against total HSV-2-infected cells (Suh et al., 1983).

As shown in Fig. 4 (lanes 1, 3 and 8), the P9 antibodies reacted primarily and specifically with both HSV-1 38K and HSV-2 36K polypeptides present respectively in extracts of HSV-1-infected BHK cells and HSV-2-infected NIH 3T3 cells. A very weak reaction with HSV-1 136K and HSV-2 138K proteins was observed, probably due to related epitopes (Cohen et al., 1986). The P9 antibodies did not react with any protein in the extracts from mock-infected NIH 3T3 or from the transformed 620-2 cells which were derived by transformation of Syrian hamster embryo fibroblasts using u.v.-inactivated HSV-2 (Kessous et al., 1979) and which displayed an up-regulated cellular protein of 38K (Suh et al., 1980; M. Suh, personal communication). Using 200 µg of the protein extracts from H2, B11G and D1G, P9 serum revealed a polypeptide (Fig. 4, lanes 5 to 7) which comigrated with the HSV-2 36K protein detected in infected NIH 3T3 and BHK cells (Fig. 4, lanes 3 and 8). Tested under the same conditions, H4, B01G, B03G and B09G cells never showed any signal; tested again with the RS21 antiserum, these clones gave the same negative results (data not shown). Thus, although they retained full-length copies of the 36K gene, the H4, B01G and B03G cell lines did not produce the viral protein. As for the B09G cell line, this lack of expression could be attributed to the partial or complete loss of the 0-6 kb band which was found to be missing by DNA analysis.

Until now, all rodent cell lines transformed with u.v.-inactivated HSV-2, the cloned BglII N fragment or its 737 bp subfragment have failed to show retention of the viral sequences (Cameron et al., 1985; Galloway & McDougall, 1981; Galloway et al., 1984). These observations have hindered investigations on the mechanism(s) of HSV-2-induced transformation because it
was not possible to identify the viral sequences and/or the cellular genes that may be involved. The transfected sequences were found stably retained in the cell lines analysed in this report (Table 1) and they were maintained unchanged in the tumour derivatives except for B01T cells where the 12 kb band was found to be missing. So far, neither of these clones have shown evidence that there was preferential loss of the viral sequences. This further supports our previous observations (Saavedra & Kessous-Elbaz, 1985) that BglII N subfragments were retained better in NIH 3T3 cells than was the entire BglII N fragment and that the absence of BglII N sequences from cells transformed by this fragment could be attributed to some poisonous sequence which selects against the BglII N-containing clones. Thus, the use of subfragments with known functions appears to be a better approach to elucidate their role in HSV-2-induced transformation. In this regard, the DNA analysis and tumorigenicity test findings demonstrate that the retention of the transfected sequences is not sufficient to produce conversion of the NIH 3T3 cell since we found almost as many tumorigenic as non-tumorigenic clones (Table 1).

For the first time, a gene mapping in the transforming BglII N fragment was found to be stably expressed in the transformed cells. Three of the seven clones that retained the transfected sequences expressed the 36K protein (Table 1); surprisingly, these are the clones (H2, B11G, D1G) with low viral DNA content and a non-resolvable 0.6 kb band. The presence of the viral product in these clones implies that the 36K coding sequences are maintained intact in the host genome. Thus, the sequences lying in the 0.6 kb band are not missing but are probably restricted with the additional bands found in their respective hybridization profile. In contrast, the lack of expression in the four remaining clones (H4, B01G, B03G, B09G) is more disturbing; it might be due to a genetic impairment in the regulatory sequences normally controlling the expression of this gene or to a negative influence of the cellular sequences flanking the integrated one. Taken together with the tumorigenicity test findings, the immunoblotting analysis shows that tumorigenic conversion of NIH 3T3 cells is not associated with 36K polypeptide expression (Table 1). This conclusion is based on the following facts: first, one of the three producing clones (B11G) does not form tumors at any of the cell concentrations tested; second, the similar amounts of the 36K product in all the expressing clones do not support the idea of a concentration-dependent effect of that protein; third, the immunoblotting assays using two different polyclonal immune sera did not reveal any remarkable change in the size of the protein or its reactivity, which could indicate that the 36K polypeptide synthesized in the tumorigenic clones has undergone structural or immunological changes. So far the tumorigenic phenotype of H2 and D1G cells does not appear to be related to quantitative or gross qualitative modifications in the viral polypeptide. This led us to conclude that the continuous expression of this protein is not implicated in BglII N-induced transformation.

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