Amplification of the Moloney murine leukaemia virus genome and its possible role in facilitation of chemical carcinogenesis in normal rat kidney cells

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In a previous study we have shown that a single infectious particle of Moloney murine leukaemia virus per cell is sufficient to facilitate chemical carcinogenesis in normal rat kidney cells. When these cells are exposed to the carcinogen after a low number of passages post-infection (p.i.), cell transformation becomes apparent only after many subsequent passages. On the other hand, when exposure is done after a high number of passages p.i., cell transformation can be detected in the treated culture or at the next passage. It is thus evident that whereas the carcinogenic effect is rapid, the viral effect becomes apparent only after a long period of latency. Here we provide evidence that this viral effect requires multiple proviruses and that the long latent period reflects the time needed for a sufficient accumulation of proviruses in some of the cells. This accumulation may result from multiple rounds of superinfection by virions released into the culture medium, although we cannot exclude other mechanisms of provirus amplification. Our data also suggest that this amplification enhances virus production.

Exogenous as well as activated endogenous non-transforming retroviruses have been shown in numerous early studies (reviewed by Tennant & Rascatti, 1980) and more recently by our laboratory (Hassan et al., 1985, 1986, 1990) to facilitate chemical carcinogenesis in mouse and rat cells. To investigate whether a single active provirus is sufficient to exert this synergistic effect, we have generated a cell clone from a single normal rat kidney (NRK) cell infected with a single infectious particle of Moloney murine leukaemia virus (M-MLV) and found that, unlike the uninfected parental NRK cells which are highly resistant to chemical carcinogenesis, this clone (NRK/MLV) can indeed be transformed by 3-methylcholanthrene (3-MC). However, we noticed that if this clone is exposed to the carcinogen after a low number (four or five) of subculture passages, cell transformation becomes evident only after nine to 11 subsequent passages, whereas if exposure is after a high number of passages (more than 20), foci of transformed cells appear in the treated culture or in the first subsequent passage (Hassan et al., 1986). It is therefore evident that whereas the effect of the carcinogen is rapid, the synergistic function of the virus becomes effective only after a long period of latency.

It is worth mentioning, in this context, the long latency for in vivo induction of neoplasms by such non-transforming retroviruses in birds (Cooper & Neiman, 1980; Hayward et al., 1981; Fung et al., 1982, 1983; Payne et al., 1982; Schubach & Groudine, 1984; Steffen, 1984), mice (Lane et al., 1982; Yoshimura & Levine, 1983; Dickson et al., 1984; Lenz et al., 1984; Nusse et al., 1984; Shen-Ong et al., 1984), rats (Tsichlis et al., 1983; Lemay & Jolicoeur, 1984) and cats (Neil et al., 1984). This induction results from integration of the viral DNA at a specific site in the cellular genome, which activates aberrant expression of a specific cellular gene by transcriptional control elements located in their long terminal repeats. Therefore, the long latency in manifesting these tumours is often attributed to the low probability of the viral DNA integrating at such a specific site, and it is postulated that many random integrations may occur during this latent period until one of them hits this specific site. In accord with this view, most of the tumours induced by retroviruses have been found to contain multiple copies of the aetiological viral genome. Therefore by analogy, we investigated whether the latency observed in the viral synergistic effect in our cells was also associated with an accumulation of multiple viral genomes.

Genomic DNA was extracted from NRK/MLV clones at the 5th [designated NRK/MLV(P-5)] and the 26th [NRK/MLV(P-26)] passages post-infection (p.i.),
Fig. 1. Southern blot analysis of DNA from transformed and untransformed M-MLV-infected NRK clones for integrated M-MLV genomes. High Mr DNA was extracted from untransformed NRK/MLV cells at the 5th [NRK/MLV(P-5)] (lane 5) and the 26th [NRK/MLV(P-26)] (lane 4) passages p.i., and from the transformed clones designated focus A (lane 3) and focus I (lane 2) from 3-MC treatment of NRK/MLV(P-5) cells, and focus Z (lane 1) from 3-MC treatment of NRK/MLV(P-26) cells. The DNA was digested with EcoRI and subjected to Southern blot analysis with an M-MLV-specific 32P-labelled probe. DNA from uninfected NRK cells served as a control (lane 6). Fragment sizes are shown (kb).

and from cloned cells of three randomly selected transformed foci. DNA was digested with EcoRI (which does not cleave within the M-MLV genome) and analysed by Southern blot hybridization to a 32P-labelled M-MLV-specific probe as previously described (Hassan et al., 1986). As can be seen in Fig. 1, the NRK/MLV(P-5) cells contained a single integrated M-MLV genome. On the other hand, the NRK/MLV(P-26) cells showed several faint, M-MLV-specific bands with varying intensity in addition to the strong band representing the original provirus which was also found in the NRK/MLV(P-5) cells. The DNA was digested with EcoRI and subjected to Southern blot analysis with an M-MLV-specific 32P-labelled probe. DNA from uninfected NRK cells served as a control (lane 6). Fragment sizes are shown (kb).

Table 1. Transformation of M-MLV-infected and uninfected NRK cells by M-MSV(124)

<table>
<thead>
<tr>
<th>Cells tested</th>
<th>Number of transformed foci†</th>
<th>Reverse transcriptase (c.p.m./10⁶ cells)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRK</td>
<td>21, 35</td>
<td>—</td>
</tr>
<tr>
<td>NRK/MLV(P-5)</td>
<td>31, 37</td>
<td>6300</td>
</tr>
<tr>
<td>NRK/MLV(P-26)</td>
<td>17, 27</td>
<td>8600</td>
</tr>
<tr>
<td>NIH/3T3</td>
<td>74, 118</td>
<td>—</td>
</tr>
<tr>
<td>NIH/3T3(MLV)</td>
<td>2, 2</td>
<td>76800</td>
</tr>
</tbody>
</table>

* Cells were plated at a density of 3 × 10⁵ per 10 cm diameter dish, and were infected for 1 h with M-MSV(124) and scored for foci of transformed cells 10 days later. Results are from duplicate cultures.
† Cells were plated at a density of 5 × 10⁵ per 5 cm diameter Petri dish in 5 ml of medium. The medium was replaced with 2.5 ml of fresh medium the following day. Viral reverse transcriptase activity was measured 4 h later as previously described (Hassan et al., 1986), and the cells were trypsinized for counting.

and infected cells are usually resistant to superinfection by the same retrovirus type (Ball et al., 1973; Fan et al., 1978). In order to evaluate superinfections in our cells, we compared the efficiency of clone 124 of Moloney murine sarcoma virus [M-MSV(124)], a pseudotype of M-MLV (Ball et al., 1973), to transform infected and uninfected NRK cells. Cells from the 5th and 26th passages p.i., and uninfected cells were infected for 1 h with 1 ml of M-MSV(124) stock at a titre of about 30 f.f.u./ml on NRK cells and about 100 f.f.u./ml on NIH/3T3 cells. A comparable number of transformed foci was found in both the infected and uninfected NRK cells at day 10 p.i. (Table 1), indicating that superinfections leading to new provirus integrations could indeed occur in the NRK/MLV cells. This lack of resistance to superinfection is most probably due to the low rate of virus production by these cells as demonstrated by measuring viral reverse transcriptase activity in the culture medium (Table 1). Indeed, M-MLV-infected NIH/3T3 [NIH/3T3(MLV)] cells, which produced virus at a much higher rate, proved to be highly resistant to superinfection (Table 1).

Three transformed foci were examined and showed multiple M-MLV-specific bands in addition to the one representing the original provirus. However, in contrast to the varying intensity of the bands of the NRK/MLV(P-26) cells, the intensity of the bands of each of these foci was the same (Fig. 1), indicating that they were monoclonal, i.e. each was derived from a single cell already containing multiple proviruses. Foci A and I were randomly chosen from passage 11 foci after 3-MC treatment of NRK/MLV(P-5) cells. They exhibited different patterns of proviral integration (Fig. 1) and it is therefore likely that they are independent clones. However, since they share four common M-MLV-
specific bands in addition to the original one found in the parental NRK/MLV(P-5) clone, we cannot exclude the possibility that they might, nevertheless, be sister clones and that their additional bands may reflect new integrations resulting from superinfections that occurred at some later time. On the other hand, focus Z appeared in a 3-MC-treated culture of NRK/MLV(P-26) cells without any further passage and was therefore certainly unrelated to either focus A or focus I. Hence, these data suggest that when the carcinogen treatment was done after a low number of passages p.i., the effect of the carcinogen was preserved in the affected cells and transferred to their descendants, although transformation was delayed until further viral DNA integration(s) occurred. With carcinogen treatment after a high number of passages however, cell transformation could be manifested without further delay since the 'complementing' integrations had apparently already occurred.

Further support for the need for reintegration comes from our previous observation that if interferon (IFN), which blocks provirus integration (Huleihel & Aboud, 1983), is added with the carcinogen at a low passage p.i., no transformation occurs as long as IFN remains in the culture (Hassan et al., 1985). However, IFN is completely ineffective in this respect when added with the carcinogen after a high number of passages p.i. Similar results have been reported by Mishra et al. (1976) with ethidium bromide or antiviral antibody; the former blocks provirus integration (Guntaka et al., 1975), whereas the latter exerts its effect probably by preventing superinfections. Nevertheless, we cannot exclude the possibility that provirus accumulation might be mediated by reverse transcription of endogenous viral RNA into viral DNA independently of exogenous superinfection, as shown in some cases (Varmus & Shank, 1976; Shen-Ong & Cole, 1984), or by transposition (Heidman et al., 1988) and other amplification mechanisms (Dudley & Risser, 1984; Colombo et al., 1988).

We compared the rate of virus release from the transformed and untransformed clones by measuring viral reverse transcriptase activity in the culture medium at several time intervals. As seen in Fig. 2, virus release was remarkably higher in the transformed clones. This might be due to the higher replication rate of these clones, the doubling time of which is 12 to 16 h compared to 32 to 48 h for the NRK/MLV(P-5) and NRK/MLV(P-26) cells. It might also reflect the increased number of proviruses per cell as presumably at least some of them were active. Although enhanced virus production was also noted in the NRK/MLV(P-26) clone, it was not as prominent as in the transformed clones, possibly because cells with multiple proviral genomes constituted only a small fraction of the cell population. Nevertheless, this does not necessarily mean that proviruses must be active in order to exert the synergistic effect with the carcinogen. As some of the M-MLV-specific restriction fragments shown in Fig. 1 are smaller than those of the complete viral genome, it is likely that some of the proviruses are defective. It is therefore possible that this effect requires only a provirus integration within a certain specific locus, and that once this integration has occurred in a certain cell, no further virus production or superinfection is needed to complement the action of the carcinogen. Interestingly, there is a virus-specific band common to all transformed clones and the NRK/MLV(P-26) cells (Fig. 1; arrow). It is tempting to regard this band as representing the required provirus integration into an effective locus, but this assumption requires more direct evidence.

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**References**


**Fig. 2.** Rate of virus release from transformed and untransformed M-MLV-infected NRK clones: NRK/MLV(P-5) (Δ); NRK/MLV(P-26) (▼); focus A (▲); focus I (▼); and focus Z (●) cells. Reverse transcriptase activity was measured in aliquots taken at the times indicated.
sis by mouse mammary tumor virus: proviral activation of a cellular

endogenous mouse mammary tumor virus genomes in mouse T-cell

infection of Moloney murine leukemia virus in mouse cells: effect on
number of viral DNA copies and virus production in producer cells.
Journal of Virology 28, 802-809.

(1982). Orientation and position of avian leukemia virus DNA relative to the cellular
oncogene c-myc in B-lymphoma tumors of highly susceptible

(1983). Activation of cellular oncogene c-erb B by LTR insertion:
molecular basis for induction of erythroblastosis by avian leukemia
virus. Cell 33, 357-368.

Ethidium bromide inhibits appearance of closed circular viral DNA
and integration of virus-specific DNA in duck cells infected with

HASAN, Y., HULEHEI, M., PRIEL, E., WOLFSON, M. & ABOUD, M.
(1985). Effect of mouse interferon on chemical carcinogenesis in
normal rat kidney cells infected with Moloney murine leukemia
virus. Carcinogenesis 6, 1787-1790.

HASAN, Y., HULEHEI, M., PRIEL, E., ROSNER, K. & ABOUD, M.
(1986). Effect of Moloney murine leukemia virus on the carcinogenic-
ity of 3-methyl-cholanthrene in normal rat kidney cells. Archives of
Virology 90, 63-71.

Chemical-retroviral carcinogenic cooperation and its molecular
basis in NIH/3T3 cells. Carcinogenesis 11, 2097-2102.

cellular oncogene by promoter insertion in ALV-induced lymphoid

gene to demonstrate intracellular transposition of defective retrovi-
uses. Proceedings of the National Academy of Sciences, U.S.A.
85, 2219-2223.

supercoiling in interferon treated cells. Journal of Virology 48,
120-126.

cellular transforming gene in tumors induced by Abelson murine

sequence homologous to a cell-virus junction fragment in several
Moloney murine leukemia virus induced rat thymomas. Proceedings of
the National Academy of Sciences, U.S.A. 81, 38-42.

LENN, J., CELANDER, D., CROUThER, R. L., PATARCA, R., PERKINS,
leukemogenicity of a murine retrovirus by sequences within the long

Chemical-viral co-carcinogenesis: requirement for leukemia virus
expression in accelerated transformation. International Journal of
Cancer 18, 852-858.

NUSSE, R., VAN OOVEN, A., COX, D., FUNG, Y. K. T. & VARMUS, H. E.
(1984). Mode of proviral activation of a putative mammary oncogene

arrangements of viral DNA and an activated host oncogene (c-myc)

London 307, 702-714.

set of intracisternal A-particle genes in mouse plasmacytoma.

SHEN-ONG, G. L. C., POTTER, M., MUSHINSKI, J. F., LAVU, S. & REDDY,
mutagenesis in plasmacytoid lymphosarcomas. Science 226,
1077-1080.

STEFFEN, D. (1984). Proviruses are adjacent to c-myc in some murine
leukemia virus-induced lymphomas. Proceedings of the National
Academy of Sciences, U.S.A. 81, 2097-2101.

TENNANT, R. W. & RASCATTI, R. J. (1980). Mechanisms of carcino-
genesis involving endogenous retroviruses. In Carcinogenesis 5.
Modifiers of Chemical Carcinogenesis, pp. 185-204. Edited by T. J.

for proviral DNA integration in MoMuLV induced rat thymic

VARMUS, H. E. & SHANK, P. R. (1976). Unintegrated viral DNA is
synthesized in cytoplasm of sarcoma virus transformed duck cells by

involving mink cell focus-inducing murine leukemia viruses have a
common region of provirus integration. Journal of Virology 45,
576-584.

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