Integration of Proviral DNA in Kirsten Murine Sarcoma Virus-infected Mouse Fibroblasts

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(Accepted 13 October 1983)

SUMMARY

The structure and sites of integration of proviral DNA were studied in 19 clonally related Kirsten murine sarcoma virus-transformed non-producer NIH/3T3 cell lines. The majority of these cell lines contained a single provirus, inserted colinearly with respect to unintegrated linear viral DNA, and lacking detectable methylation at MspI/HpaII sites. Although all proviruses were located at distinct integration sites in the host cell genome, the possible existence of similarities between some adjacent host flanking sequences, suggested from restriction mapping data, could not be ruled out. In three phenotypically reverted cell lines no change in either proviral DNA or adjacent host flanking sequences was detectable. In addition, the revertant proviruses lacked detectable methylation at MspI/HpaII sites. These findings suggest that changes in cellular function(s) may be responsible for loss of transformed phenotype in these cells.

INTRODUCTION

Following infection of a host cell, the single-stranded RNA genome of a retrovirus is reverse-transcribed into double-stranded DNA (proviral DNA) which becomes covalently integrated into cellular DNA (Varmus & Swanstrom, 1982; Varmus, 1982). Kirsten murine sarcoma virus (KiMSV) is a strongly oncogenic defective retrovirus, derived from the weakly oncogenic Kirsten murine leukaemia virus (KiMLV) and sequences transduced from the rat genome (Kirsten & Mayer, 1967; Anderson & Robbins, 1976; Shih et al., 1978). Extensive molecular analyses of KiMSV proviral DNA have shown it to contain long terminal repeats (LTRs) similar to those of other retroviruses (Tsuchida et al., 1981; Norton et al., 1982). These LTRs are derived from the progenitor KiMLV genome (Norton & Avery, 1982) whereas a long 5 kbp internal region of KiMSV DNA is composed of rat-derived sequences, a part of which encodes the viral transforming protein (Shih et al., 1978; Ellis et al., 1981; Tsuchida et al., 1982).

We have previously described the isolation and biological characterization of a clonally related series of KiMSV-infected mouse fibroblasts, derived from a cloned NIH/3T3 cell line (Morris et al., 1980). These comprise a number of transformed, non-producer cell lines obtained by low-multiplicity infection and a series of three phenotypically untransformed variants (revertants) derived independently from one parental transformed cell line. All three revertants were shown to have retained a sarcoma provirus that could be rescued by superinfection with a non-defective murine leukaemia (helper) virus (Morris et al., 1980). Therefore these cells provide an opportunity to study the integrated proviral DNA of a highly oncogenic murine virus in a related series of transformed cells, and in cells which have reverted to a normal phenotype.

In this report, we describe the analysis of the structure and methylation status of proviral DNA in non-producer transformed and revertant cells, by restriction mapping using the Southern (1975) blotting technique. We also describe a comparison of the proviral integration sites in the DNA of the various cell lines using the same methods.

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0022-1317/84/0000-5811 $02.00 © 1984 SGM
METHODS

Cells and viruses. NIH/3T3 cells were originally obtained from Dr J. Levy (University of California, San Francisco, Ca., U.S.A.) and cloned in our laboratory (Morris et al., 1980). KiMSV (MLV), supplied as a culture of infected rat kidney cells (KNRK) producing $8 \times 10^4$ p.f.u and $1 \times 10^6$ focus-forming units (f.f.u.) was generously provided by Dr S. Aaronson (NIH, Bethesda, Md., U.S.A.). The non-producer cell lines used in this study have been described by Morris et al. (1980).

Restriction enzyme digestion and agarose gel electrophoresis. High molecular weight DNA was extracted as described previously (Avery et al., 1980) and digested with an excess of each restriction enzyme (New England Biolabs) under the conditions recommended by the suppliers. SDS was then added to 0.5% (w/v) and the digested DNA was electrophoresed on a horizontal agarose slab gel in 40 mM-Tris, 20 mM-sodium acetate, 2 mM-EDTA (adjusted to pH 7.7 with acetic acid). The sizes of restriction fragments were determined relative to EcoRI (Thomas & Davis, 1975) and HindIII (Wellauer et al., 1974) fragments of bacteriophage lambda DNA.

DNA probes and filter hybridization. A 4.6 kbp cloned DNA fragment (designated ‘KSma’: see Norton & Avery, 1982) was used as a representative probe. To detect proviral restriction fragments representing the 5' side of the genome, a 0.5 kbp TaqYI fragment (Norton et al., 1982) was obtained by preparative electrophoresis after TaqYI digestion of the KSma clone. DNA was labelled to a specific activity of $10^8$ ct/min per µg by the nick translation procedure essentially as described by Rigby et al. (1977).

After electrophoresis, DNA was transferred to nitrocellulose filters (BA 85, Schleicher & Schüll) as described by Southern (1975) and annealed with labelled DNA (3 × 10^6 ct/min per filter) exactly as described previously (Norton et al., 1982). Filters were washed for 2 h in 300 mM-NaCl, 30 mM-trisodium citrate at room temperature, then in 15 mM-NaCl, 1.5 mM-trisodium citrate at 65 °C for 20 min. Autoradiography was performed at -70 °C for 1 to 14 days using a DuPont Cronex intensifying screen.

RESULTS

KiMSV proviral DNA in non-producer cells

Uninfected NIH/3T3 cell DNA contains multiple endogenous proviral sequences closely related to MLV genomes and to the MLV-derived sequences of KiMSV DNA (Steffen & Weinberg, 1978; Steffen et al., 1982; Avery et al., 1980). In order to detect restriction fragments containing KiMSV proviral DNA against this background of related sequences, a molecularly cloned probe (KSma) specific for the rat-derived sequences of the KiMSV genome was employed (Norton & Avery, 1982). This probe hybridized to a small extent with two classes of sequences in uninfected mouse DNA. The most prominent hybridization was with the mouse homologue of the viral 'Kras' oncogene (see Ellis et al., 1981). The second source of background hybridization represented weak homology between the probe and multiple endogenous, virus-like '30S' genes present in mouse DNA (Shih et al., 1978; Courtney et al., 1982).

Using the KSma sequence probe and several restriction enzymes we have surveyed the proviruses in a total of 19 non-producer cell lines. The data obtained are summarized by the restriction maps in Fig. 3. However, for the sake of brevity we present only representative results in this section.

The enzymes PstI and KpnI cleave linear unintegrated KiMSV proviral DNA at sites within the LTRs (Tsuchida et al., 1981; Norton et al., 1982) as shown in Fig. 3. Therefore these enzymes can be used to examine the internal organization of integrated proviruses. In this way proviral sequences extending into both 3' and 5' LTRs were shown to be invariably present in non-producer cell DNA. The sequence organization found also indicated a colinear insertion of the provirus into host cell DNA (data not shown).

Analysis of adjacent host flanking sequences was achieved using the enzymes HindIII, EcoRI and BamHI. These generate restriction fragments containing both viral and adjacent cell sequences (junction fragments). Fig. 1(a) shows the results of HindIII digestion of DNA from several non-producer cell lines. Some hybridization of the probe to uninfected NIH/3T3 cell DNA is evident (lane 1) but this does not seriously impede interpretation of the digest patterns. The internal 1.6 kbp fragment was generated from all the cell DNAs in Fig. 1, in common with all other non-producer DNAs examined. In most cases two additional (junction) fragments were produced, implying the presence of a single provirus. However CD11 DNA yielded a total of four junction fragments (Fig. 1a, lane 3) implying the existence of two proviruses. Analyses with
Integration of KiMSV DNA

Fig. 1. Analysis of KiMSV-transformed and revertant non-producer cell DNAs with HindIII. 10 µg samples of cell DNA were digested to completion, electrophoresed on a 1.0% agarose gel and the separated fragments transferred to a nitrocellulose filter. This was hybridized with 32P-labelled KSma probe (see Methods), washed and then autoradiographed. (a) DNA from cell lines (1) uninfected NIH/3T3, (2) CG11, (3) CD11, (4) CH6, (5) DG4 and (6) CBg. (b) DNA from cell lines (1) CC1, (2) R1-1, (3) R2-1 and (4) R5-5-1.

Fig. 2. Analysis of KiMSV-transformed cell DNAs with EcoRI. EcoRI digests of DNA from the following non-producer cell lines (1) uninfected NIH/3T3, (2) DA4, (3) CA5, (4) CA9, (5) CA12, (6) CB6, (7) DH9 and (8) CA11 were electrophoresed on a 0.8% gel and fragments containing KiMSV DNA were detected as described in the legend to Fig. 1.

PstI and KpnI showed one of these proviruses to possess a deletion (see Fig. 3). As can be seen in Fig. 1 (a) the sizes of the HindIII junction fragments are different in each cell line indicating that different host cell DNA sequences flank each provirus.

Fig. 2 shows the results obtained from analysis of another series of non-producer cell DNAs with the enzyme EcoRI, which cuts the KiMSV provirus once (see Fig. 3). As with HindIII digestion, two junction fragments were detected in most cell lines examined, indicating the
Fig. 3. Restriction maps of KiMSV proviral DNA and adjacent host flanking sequences. Proviral DNA (6.5 kbp) is indicated by long open boxes in each non-producer cell line. In CB8 and CD11 the solid boxes show the size of the deletions which were localized to the regions enclosed by the bars. Only some cell DNAs were analysed with BamHI; absence of a BamHI site indicates that the DNA was not tested with this enzyme. A map of sites in proviral DNA is shown at the bottom of the figure (from Norton et al., 1982) where the open boxes represent the long terminal repeats on the DNA. H, HindIII; R, EcoRI; P, PstI; B, BamHI; K, KpnI; S, SmaI.
presence of a single provirus. Moreover, the sizes of these junction fragments were again
different in each cell line. DH9 DNA (Fig. 2, lane 7) gave rise to a more complex pattern of
EcoRI fragments, a total of five bands being visible. We have not attempted to define further the
organization of proviral and host flanking sequences in this cell line and have simply classified it
as containing ‘multiple’ proviruses.

In the three revertant cell lines (R1-1, R2-1 and R5-5-1) no alteration in either the internal
organization of the provirus or adjacent host flanking sequences was found as compared to the
parental transformed cell line, CC1. For example, Fig. 1(b) shows a HindIII digestion of the
revertant cell and CC1 DNAs, all of which can be seen to contain a single provirus.

Out of a total of 19 non-producer cell DNAs-studied, the majority (14) contained a single
provirus. Three (DH9, CD12 and DA12) containing multiple proviruses were not amenable to
further analysis and two (CB8 and CD11) were identified as harbouring a deleted provirus in
addition to a single intact viral genome. Only one example of a cell line containing a single
deleted provirus (CB3) was found. We have reported a more extensive characterization of this
deletion elsewhere (Clewley et al., 1983).

As previously indicated, the complete results of our study are summarized in Fig. 3, where
restriction maps for those cell lines containing a single provirus are shown. The orientation of
the various fragments was determined using a probe specific for the 5′ side of the provirus (see
Methods). It should be noted that this method yields only a partial restriction map, since only
those sites for each enzyme nearest the provirus are detectable. Although all proviruses appear
to be integrated at different sites in the host cell DNA, inspection of Fig. 3 does reveal possible
similarities between some integration sites. For example, the locations of EcoRI and HindIII
sites on the 3′ side of the CB9 provirus are very similar to those on the 3′ side of the CA11
provirus. Also, the 3′ EcoRI and HindIII sites flanking the CB6 and DH1 proviruses appear very
similar.

Fig. 3 also shows the size and approximate location of deletions found in association with
intact proviruses in the cell lines CB8 and CD11. We have inferred the structures of these
deletions as the simplest interpretation of the data and we cannot exclude the possible presence
of non-viral sequences and/or rearrangements in these proviruses.

Methylation status of proviral DNA

The enzyme MspI, which recognizes the sequence CCGG, cleaves KiMSV DNA at a number
of sites such that the KSma probe can be used to detect four internal fragments by Southern blot
analysis (unpublished observations). However, the MspI isoschizomer HpaII cannot cleave the
CCGG recognition sequence when the internal C residue is methylated (see Van der Ploeg &
Flavell, 1980). Therefore these two enzymes were used to investigate the methylation status of
proviral sequences in non-producer cell DNA as shown in Fig. 4.

When uninfected NIH/3T3 cell DNA was cleaved with MspI (Fig. 4, lane 1) and hybridized
with KSma probe, several fragments larger than 7 kbp were detected. These hybridizing
sequences represent those components of mouse DNA mentioned previously which show
homology to the KSma probe. MspI digestion of DNA from a non-producer cell line (CC1),
produced in addition to these bands four internal KiMSV-specific fragments ranging in size
from 1·6 to 0·5 kbp (lane 3) although the 0·5 kbp fragment is poorly visible in the figure. When
CC1 DNA was cleaved with HpaII, the same internal fragments were detected (lane 4) at a level
comparable to that when MspI was used. This shows that the proviral DNA is essentially
unmethylated at least at the MspI/HpaII sites. By contrast, HpaII did not cleave in and around
the KSma-related endogenous sequences in either uninfected mouse DNA (lane 2) or in the CC1
DNA (lane 4). We have also found no change in methylation status of endogenous MLV-related
proviral sequences in non-producer cell DNA (unpublished observations). Thus, KiMSV
transformation is not associated with demethylation of normal mouse DNA sequences. Several
other non-producer cell DNAs, each containing a single provirus, gave identical results to those
shown for CC1 (data not shown). Therefore KiMSV proviruses located at various sites in the
host chromosome appear to lack detectable methylation at their MspI/HpaII sites.
Fig. 4. Methylation status of proviral DNA. 10 μg samples of DNA were digested with a sixfold excess of either \textit{Mspl} (lanes 1, 3, 5, 7 and 9) or \textit{HpaII} (lanes 2, 4, 6, 8 and 10) and electrophoresed on a 1.2% gel. Fragments containing KiMSV-related sequences were detected as described in Fig. 1. Samples 1 and 2, uninfected NIH/3T3 DNA; 3 and 4, CC1 DNA; 5 and 6, RI-1 DNA; 7 and 8, R2-1 DNA; and 9 and 10, R5-5-1 DNA.

When DNA from the three revertant cell lines was analysed with \textit{Mspl} the same four internal proviral fragments were found as in the parental transformed line, CC1 (Fig. 4, lanes 5, 7 and 9). Similarly, \textit{HpaII} digestion generated internal fragments of intensity comparable to those produced by \textit{Mspl} in each of the revertant cell DNAs (lanes 6, 8 and 10). Therefore we can conclude that the revertants are identical to the parental transformed line in lacking methylation at their proviral \textit{Mspl/HpaII} sites.

\textbf{DISCUSSION}

Previous studies on the integration of various retrovirus proviruses have revealed no evidence for specific or preferential integration at particular chromosomal sites (Hughes \textit{et al.}, 1978; Steffen & Weinberg, 1978; Bacheler & Fan, 1979; Lerner \textit{et al.}, 1981). Although other workers have previously investigated murine retrovirus integration in mouse cells (Bacheler & Fan, 1979, 1981; Van Beveren \textit{et al.}, 1982) the results presented in our study represent the first detailed systematic survey of provirus integration with a strongly oncogenic murine retrovirus in the mouse cell.

Of the 19 clonally related KiMSV-transformed non-producer cell lines examined here all contained provirus(es) integrated colinearly with respect to unintegrated linear viral DNA (similar to retroviruses described by other workers). We have found no instance of any two independent infection events resulting in two proviruses being located at identical sites in the host chromosomal DNA. We have also found no evidence for specific integration sites in cells infected at high multiplicity with KiMSV (J. Norton, unpublished results). These observations are of particular importance since uninfected mouse DNA contains multiple endogenous proviral sequences closely related to MLV sequences (and to the corresponding LTR regions of KiMSV DNA) which offer the potential for homologous integrative recombination events.

On the basis of restriction enzyme sites, some similarities in cell DNA sequences flanking different proviruses were observed. In other studies the occurrence of similar-sized proviral junction fragments has been taken as evidence for possible 'targeting' of provirus integration.
Integration of KiMSV DNA (Cohen & Murphey-Corb, 1983). It is possible that the similarities in sizes of different junction fragments observed here may genuinely reflect similarities in sequence or alternatively they could be merely coincidental. A more detailed analysis by the methods employed here would be unlikely to distinguish between these possibilities, since the regions of putative flanking sequence homology may not be amenable to comparison by restriction mapping. Analysis of molecularly cloned junction fragments would be required to resolve this issue.

In two non-producer cell lines containing a deleted provirus in addition to an intact viral genome we note that the deletions are similarly located and occur in that region of the genome encoding the transforming protein of the virus (Ellis et al., 1981; Tsuchida et al., 1982). It seems plausible that these deleted proviruses may lack a competent transforming gene, in contrast to another deleted KiMSV genome we have examined (Clewley et al., 1983). The occurrence of these deletions in association with an intact provirus in the same cell may be rationalized on the basis that the non-producer cell lines were selected by the criterion of being transformed (Morris et al., 1980).

We have examined the methylation status of transcriptionally active KiMSV proviruses located at different chromosomal sites in a number of non-producer cell lines. It is generally found that actively transcribing genes are undermethylated compared to inactive genes (for review, see Doerfler, 1981) and the same appears to be true for retrovirus proviruses (Guntaka et al., 1980; Weinberg & Steffen, 1981; Montandon et al., 1982; Hoffmann et al., 1982). However, it is not known to what extent the site of integration can influence the methylation status of proviral DNA. All KiMSV proviruses examined were found to lack detectable methylation at their MspI/HpaII sites. Although only a limited number of cell lines were studied, our data would support the notion that the methylation status of exogenously acquired proviral DNA is independent of the integration site in the cellular DNA.

In three revertant cell lines, each isolated independently from a single parental transformed line, no change in either proviral or adjacent host flanking sequences was found. This is in marked contrast to other revertants of KiMSV-transformed cells that have been described where the provirus has been lost (Trainor & Reitz, 1979). In addition, the revertants were found to be identical to the parental transformed line in lacking methylation at their proviral MspI/HpaII sites. Taken together with the observation that a transforming virus can be rescued from these cells (Morris et al., 1980), our findings suggest that the revertants more closely resemble those described by Stephenson et al. (1973). Thus the reversion may involve an alteration in cell-encoded function(s) required for transformation.

We thank Ms Jane Connor and Ms Janis Wignall for excellent technical assistance and the Cancer Research Campaign for generously supporting this work.

REFERENCES


(Received 20 June 1983)