Triple basepair changes within and adjacent to the conserved YY1 motif upstream of the U3 enhancer repeats of SL3-3 murine leukemia virus cause a small but significant shortening of latency of T-lymphoma induction

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Abstract

A highly conserved sequence upstream of the transcriptional enhancer in the U3 of murine leukemia viruses (MLVs) was reported to mediate negative regulation of their expression. In transient expression studies, negative regulation was reported to be conferred by coexpression of the transcription factor YY1, which binds to a motif in the upstream conserved region (UCR). To address the function of the UCR and its YY1-motif in an in vivo model of MLV-host interactions we introduced six consecutive triple basepair mutations into this region of the potent T-lymphomagenic SL3-3 MLV. We report that all mutants have retained their replication competence and that they all, like the SL3-3 wild type (wt), induce T-cell lymphomas when injected into newborn mice of the SWR strain. However, all mutants induced disease with slightly shorter latency periods than the wt SL3-3, suggesting that the YY1 motif as well as its immediate context in the UCR have a negative effect on the pathogenicity of the virus. This result may have implications for the design of retroviral vectors.

Keywords: Murine leukemia virus; YY1; Upstream conserved region; SL3-3; Lymphomagenicity

Introduction

Murine leukemia virus (MLV) isolates may induce a variety of tumors in the hematopoietic compartment when injected into newborn mice of inbred strains. For prototypic, potent disease-inducing viruses, such as Moloney, Friend, and SL3-3 MLVs, critical determinants of disease induction have been assigned to a region of U3 termed the transcriptional enhancer (Chatis et al., 1984; DesGroseillers and Jolicoeur, 1984; Lenz et al., 1991). The enhancer comprises direct tandem repeats with densely packed recognition motifs for several cellular transcription factors, and a number of studies have further determined a critical function of many of these motifs for the pathogenicity and specificity of the virus (Ethelberg et al., 1997b; Hallberg et al., 1991).

However, not only the enhancer sequences but also sequences upstream of the enhancer (Baum et al., 1995; Baum et al., 1997; Flanagan et al., 1989; Wahlers et al., 2002b) or between the enhancer and the promoter (Hanecak, Patten-gale, and Fan, 1991; Ishimoto et al., 1987; Laimins et al., 1984; Li et al., 1987; Tupper et al., 1992) may serve to
modulate the transcriptional activity of the virus in a general or tissue-specific manner. Few reports have addressed the role of such cis-acting sequences in models of MLV disease induction (Ishimoto et al., 1987; Tupper et al., 1992; Yoshimura and Diem, 1995).

We here look into the role of the upstream conserved region (UCR) (AGTAACGCCCATTTTGCAAGG) (Fig. 1), which is highly conserved among MLV isolates (Golemis, Speck, and Hopkins, 1990). The UCR sequence was first reported to negatively regulate expression from the endogenous B34 MLV LTR (Flanagan et al., 1989). Subsequently, the UCR sequence was used as a DNA probe to isolate the UCR binding protein (UCRBP), now commonly referred to as YY1. YY1 was found to mediate negative regulation of reporter gene transcription driven from a Moloney MLV LTR in transient expression assays (Flanagan et al., 1992). The reported negative effect of the YY1 site on transcription driven by a Moloney MLV LTR has an impact on the design of retroviral vectors. The widely used MND vector, which is based on the myeloproliferative sarcoma virus, harbors a deletion of a fragment encompassing the UCR (Challita et al., 1995). This deletion made the MND vector superior to its parental construct with respect to expression in embryonal carcinoma cells (Osborne et al., 1999). However, it was not investigated to what extent this difference was caused by removal of the YY1 motif or by other cis-elements. A recent study has investigated the role of the UCR and flanking sequences for expression of retroviral vectors based on the myeloproliferative sarcoma virus or the polycythemic strain of spleen focus-forming virus (Wahlers et al., 2002a). It was found that deletion of a 59-bp fragment (from +34 to +94 of the provirus sequence) caused about a twofold reduction of expression rates in the lymphoid and hematopoietic cell lines investigated. This reduction could, at least in part be attributed to the binding site for NFAT (nuclear factor of activated T cells) within the deleted region downstream of the YY1 motif (Rao, Luo, and Hogan, 1997). Sequence comparison (Fig. 1B) indicates

Fig. 1. Map of UCR structure and introduced mutations. (A) Schematic organization of the U3 region of SL3-3 LTR, including the UCR and the enhancer repeats (2 × 72 bp + 34 bp). Primers used for amplification of the U3 region are indicated by arrows. Forward primer: 5’-TTCATAAGGCTTTAGCAGCTA-3’; reverse primer: 5’-GGTTGATCCCCGGTCATCTGGG-3’. (B) Sequence comparison of UCRs from different retroviral LTRs. Indicated by vertical lines are regions harboring the consensus sequences of the YY1 motif (CGCCATTTT), C/EBPβ motif (TTTTGCAAG), Ikaros motif (ATGG-GAAAAA), NFAT motif (TGGAAA), ELP motif (CAGATCAAGGTCAG), SRY motif (AAAAACAAAGAC), and Sp1/EGR1 motif (AGGGCGGGT). The two point mutations found in some of the provirus sequences of SL3-3 LTR are marked with an arrow (positions 85 and 99). The C/EBPβ and SRY motif were identified by MatInspector V2.2 (http://transfac.gbf.de) (C) The UCR sequence of SL3-3 wt and the six mutant viruses, with the UCR core motif underlined. The triple basepair mutations are indicated in bold.
that this NFAT binding site may be absent from the LTR of the endogenous MLV of the BALB/c strain used by Flanagan et al. (1989) (Flanagan et al., 1992). Hence, although these studies of retroviral vectors indicate that U3 sequences upstream of the enhancer may have positive as well as negative effects on expression they do not assign specific functions to the YY1 motif. The reported negative effect of the UCR and its YY1 motif may seem contradictory to its strong conservation among MLVs, in particular since molecular evolution toward more potent viruses is commonly observed in mouse models of MLV pathogenicity. However, the possibility remains that the negative effect results from specialized conditions of the assay system, including the cell cultures used. Alternatively, the function of the YY1 motif may depend on the wider context of the LTR and hence differ among strains of MLV. Conceivably, such context-dependent negative effects may be more pronounced for LTRs endogenous to the mouse genome than for LTRs of viruses selected for tumorigenic properties.

To investigate the role of the UCR and its YY1-motif in an in vivo model of MLV–host interactions we have chosen the potent T-lymphomagenic SL3-3 MLV. The LTR of SL3-3 is more closely related to the endogenous LTR used for assigning a negative function to the UCR than are the other prototypic, potently pathogenic MLVs. We have introduced six consecutive triple basepair mutations within and adjacent to the YY1 motif in the UCR of this virus. We here report that all mutants have retained their replication competence and that they all, like the wild-type (wt) SL3-3 induce T-cell lymphomas when injected into newborn mice of the SWR strain. However, all mutants induced disease with slightly shorter latency periods than the wt SL3-3, suggesting the YY1 motif as well as its immediate context in the UCR has a negative effect on the pathogenicity of the virus.

Results

Generation of SL3-3 viruses with mutations in the YY1 motif of the UCR

Six mutants were generated to investigate the role of the YY1 binding site for the lymphomagenicity of SL3-3. The mutations were designed on basis of the YY1-binding studies done by Flanagan et al. (1992). Using the exact same nucleotide changes and nomenclature (Fig. 1C) the mutants ucrSK328, ucrSK330, ucrSK332, ucrSK334, ucrSK336, and ucrSK338 were designed. Among these, ucrSK330, ucrSK332, and ucrSK334 were reported to be critical for binding, in agreement with later studies of the DNA-binding specificity of YY1 (Flanagan et al., 1992; Hyde-DeRuyscher, Jennings, and Shenk, 1995).

Pathogenicity of UCR mutants

The pathogenicity of the mutants was analyzed in the inbred strain SWR, which does not harbor any endogenous ecotropic proviruses. All viruses caused development of disease within 4 months (Fig. 2). All the tumors induced were lymphomas and with primary manifestations in spleen, thymus, and lymph nodes, conforming to previous findings for SL3-3-induced tumors (Ethelberg et al., 1997c; Nieves, Levy, and Lenz, 1997). The incidences and latency periods of disease development are given in Table 1. The SL3-3 wt caused malignant lymphomas in all infected mice, with the
Ig

Rearrangements were detected by hybridization with the given in Fig. 3 and the results are summarized in Table 1. Some of the tumors. Examples of the hybridization data are cases, whereas J1 rearrangements could be seen in only tumors. The relative number of tumors harboring proviruses with an altered number of enhancer repeats did not appear to differ among the six UCR mutants or the wt. Moreover, the sequence analysis also demonstrated that duplications appear to occur far more frequently than deletions, as previously reported in SL3-3 induced tumor in NMRI mice (Ethelberg et al., 1999).

Molecular characterization of proviruses in tumor DNA

To detect integrated proviruses the Southern filters were hybridized with an ecotropic-specific probe (Sorensen et al., 1996). With a few possible exceptions, one to several proviruses were detected in all tumors induced by wt and mutant viruses (Fig. 3B). To test whether the SL3-3 viruses with mutated UCR underwent reversion or other mutational changes during the development of tumors, genomic DNA from tumor tissues was used as template for PCR amplification of the U3 regions from proviruses in tumors, using the primer pair shown in Fig. 1A. All the PCRs generated the expected 369-bp fragment (data not shown). In addition, products larger or smaller than the 369-bp fragment were also generated. Sequence analysis showed that most of the additional fragments harbored duplications or deletions of the tandem 72-bp repeats as previously reported for tumors induced by SL3-3 (Ethelberg et al., 1997a, 1997b, 1997c; Morrison, Soni, and Lenz, 1995; Nieves, Levy, and Lenz, 1997). Of all the 96 tumors subject to the analysis, 43% harbored a duplication of one of the 72-bp repeats, and a number of other types of duplications and deletions were also detected in some of the tumors. The relative number of tumors harboring proviruses with an altered number of enhancer repeats did not appear to differ among the six UCR mutants or the wt. Moreover, the sequence analysis also demonstrated that duplications appear to occur far more frequently than deletions, as previously reported in SL3-3 induced tumor in NMRI mice (Ethelberg et al., 1999).

Discussion

We have addressed the function of the YY1 motif and flanking sequences of the UCR of MLV in a pathogenesis model of induction of T-lymphomas by SL3-3 MLV. In case of transcription factor sites within the enhancer it has been amply demonstrated that the alteration of just a few nucleotides of one recognition site may affect the tumorigenic potency of the virus. Tumor induction may in fact provide a sensitive assay for the contribution of individual sites, whose effects may be amplified during multiple rounds of replication. Moreover, insertional activation of

Table 1

Induction of lymphomas in SWR mice by SL3-3 wt and mutant UCR virus

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. of mice with malignant lymphomas/ no. of mice analyzed</th>
<th>Mean latency period (days)</th>
<th>P value of log rank test</th>
<th>TCR-β Rearrangements/ tumors analyzed</th>
<th>Igκ Rearrangements/ tumors analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL3-3 wt</td>
<td>9/9</td>
<td>107 ± 29</td>
<td>0.000</td>
<td>5/5</td>
<td>0/5</td>
</tr>
<tr>
<td>ucrSK328</td>
<td>10/11</td>
<td>93 ± 22</td>
<td>0.0198</td>
<td>10/10</td>
<td>0/10</td>
</tr>
<tr>
<td>ucrSK330</td>
<td>20/20</td>
<td>80 ± 19</td>
<td>0.0278</td>
<td>10/10</td>
<td>0/10</td>
</tr>
<tr>
<td>ucrSK332</td>
<td>23/27</td>
<td>82 ± 16</td>
<td>0.0183</td>
<td>10/10</td>
<td>0/10</td>
</tr>
<tr>
<td>ucrSK334</td>
<td>18/18</td>
<td>84 ± 39</td>
<td>0.015</td>
<td>10/10</td>
<td>0/10</td>
</tr>
<tr>
<td>ucrSK336</td>
<td>21/21</td>
<td>77 ± 18</td>
<td>0.0164</td>
<td>6/6</td>
<td>0/6</td>
</tr>
<tr>
<td>ucrSK338</td>
<td>19/19</td>
<td>84 ± 18</td>
<td>0.0147</td>
<td>6/6</td>
<td>0/6</td>
</tr>
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</table>

longest latency period of 107 days within the series of viruses. All mice injected by the mutant viruses developed malignant lymphomas. The mean latency period to lymphoma development was shorter for all the mutants than for the wt SL3-3. According to the log rank test the shortening of latency period compared to the wt was significant for all six mutants (Table 1). Among the mutants, no significant differences were observed.

Molecular characterization of lymphomas

By histologic examination all tumors from this study were found to be large-cell lymphoblastic malignant lymphomas. No differences in tumor types induced by the SL3-3 wt and the six UCR mutants were found. To test whether the tumors were T-cell or B-cell lymphomas, Southern blotting analysis was done as previously described (Ethelberg et al., 1997a). All the 59 tumors randomly chosen as representatives of different series of the mutants and wild type were found to contain rearrangements of the TCR-β chain locus. J2 rearrangements were detected in all cases, whereas J1 rearrangements could be seen in only some of the tumors. Examples of the hybridization data are given in Fig. 3 and the results are summarized in Table 1. No rearrangements were detected by hybridization with the Igκ probe (Fig. 3, Table 1), verifying that all tumors were T lymphomas.

Molecular characterization of proviruses in tumor DNA

To detect integrated proviruses the Southern filters were hybridized with an ecotropic-specific probe (Sorensen et al., 1996). With a few possible exceptions, one to several proviruses were detected in all tumors induced by wt and mutant viruses (Fig. 3B). To test whether the SL3-3 viruses with mutated UCR underwent reversion or other mutational changes during the development of tumors, genomic DNA from tumor tissues was used as template for PCR amplification of the U3 regions from proviruses in tumors, using the primer pair shown in Fig. 1A. All the PCRs generated the expected 369-bp fragment (data not shown). In addition, products larger or smaller than the 369-bp fragment were also generated. Sequence analysis showed that most of the additional fragments harbored duplications or deletions of the tandem 72-bp repeats as previously reported for tumors induced by SL3-3 (Ethelberg et al., 1997a, 1997b, 1997c; Morrison, Soni, and Lenz, 1995; Nieves, Levy, and Lenz, 1997). Of all the 96 tumors subject to the analysis, 43% harbored a duplication of one of the 72-bp repeats, and a number of other types of duplications and deletions were also detected in some of the tumors. The relative number of tumors harboring proviruses with an altered number of enhancer repeats did not appear to differ among the six UCR mutants or the wt. Moreover, the sequence analysis also demonstrated that duplications appear to occur far more frequently than deletions, as previously reported in SL3-3 induced tumor in NMRI mice (Ethelberg et al., 1999).

Importantly, complete sequence analysis of a total of 135 nonrearranged and rearranged bands of wt and mutant viruses revealed no cases of nucleotide alterations of the introduced mutations. Moreover, very little sequence variation was observed upstream of the enhancer for both wt and mutant viruses. Based on this analysis of 135 bands derived from 95 tumors, nucleotide alterations were observed at only two positions [85 (C to T) and 99 (A to C), Fig. 1C] between the YY1 motif and the enhancer. These changes were seen as a mixture with the parental sequence and were detected frequently in bands of both wt and mutant. Our data do not allow us to assign any significance to these changes.
oncogenes may be sensitive to enhancer strength. In addition, less understood aspects of the disease process, such as apoptosis caused by viral infection, may depend upon viral replication kinetics and enhancer strength in a complex manner.

Our results show that mutations that will disrupt the binding of YY1 to its target DNA caused a shortening of the mean latency period of disease induction by 15–25 days as compared to the latency period for the wt SL3-3 of 107 days. This shortening is significant and suggests that binding of YY1 to the UCR may have a negative effect on disease induction. However, the control mutations introduced in sequences adjacent to the YY1 binding site had a similar impact on disease induction. Although there was no significant difference among the six mutants the latency periods for disease induction were shorter than that observed for the wt in all cases. Hence, the effect on disease induction does not correlate with the predicted change in the binding of YY1 to DNA as observed by in vitro studies (Flanagan et al., 1992).

We must therefore propose more complex or alternative mechanisms to explain our results. One possibility is that all mutations reduce the binding of a protein complex consisting of YY1 as well as other proteins. Such a complex may recognize nucleotides within as well as adjacent to the YY1 motif. Although we have no clues as to the nature of such a complex, we note that YY1 function may be mediated by association with other proteins (Thomas and Seto, 1999). The DNA-bending properties of YY1 may contribute to the overall function, promoting or preventing the binding of nearby transcription factors to nearby binding sites (Ayuk et al., 1999; Rao, Luo, and Hogan, 1997). An alternative explanation is that we have hit a binding motif that recognizes another protein or protein complex that does not contain YY1. In the context of SL3-3 LTR, several transcription factor binding sites (Fig. 1B) are either overlapping with (C/EBPβ) or located immediately downstream (Ikaros) of the YY1 binding site, which determines a complex environment for delineating the exact roles of YY1 in SL3-3 transcription.

Transcriptional silencing has been a significant obstacle to achieving persistent expression from retroviral vectors in vivo. Studies have shown that multiple factors may contribute to this process, one of which is the viral DNA sequences, including the UCR. By using an assay of vector silencing in transplanted fibroblasts, it was recently reported that either the disruption or deletion of the UCR resulted in the relief of transcriptional silencing (Prasad Alur et al., 2002). Another recent report addressed the role of the UCR for expression of retroviral vectors in hematopoietic cell

Fig. 3. Molecular characterization of tumors by Southern blotting analysis. Genomic DNA from tumors was digested with HindIII, resolved on a 0.8% agarose gel, blotted onto a membrane, and hybridized with probes as indicated under each blot (J1 and J2; TCR), Igk, and Eco. (A) Genomic DNA prepared from SL3-3 wt-induced tumors (lane 1–5). Control DNAs (C S and C B ) were isolated from spleens of mock-injected SWR and BALB/c mice, respectively. Arrows indicate sites of unrearranged germ line fragments (J1, J2, and Igk). (B) Genomic DNA prepared from mutant- (ucrSK328) induced tumors (lane 1–10). Arrows and size markers as in A.
lines (Wahlers et al., 2002a). Although this latter work identified a positive role of the NFAT motif of myeloproliferative sarcoma virus and Friend spleen focus-forming virus, the role of the YY1 motif was not directly addressed. Still another report has demonstrated that the Ikaros binding motif in the enhancer region upregulates the transcription from the U3 region of MCF 247 (DiFronzo et al., 2002). However, the function of the Ikaros motif in the upstream region remains to be elucidated.

All SL3-3 mutant viruses analyzed in the present study resulted in increased pathogenicity in mice, indicating that the mutated UCR may contribute to relief from transcriptional silencing by an unknown mechanism, to an increase in proviral enhancer strength, or a combination hereof. Our results suggest that the YY1 motif and its immediate context may be relevant targets for optimizing the LTRs of retroviral vectors toward improved function in T-lymphoid cells.

Materials and methods

Generation of viral mutants

Mutations were introduced into a molecular clone of SL3-3 by oligonucleotide directed mutagenesis as described (Hallberg et al., 1991; Lovmand et al., 1998), and U5-U3 and R-regions were confirmed by sequence analysis. All six mutant viruses replicated on NIH 3T3 fibroblasts as judged by reverse transcriptase assays. Polymerase chain reaction (PCR) sequence analysis of the SL3-3UCR in the resulting cultures (using the primers shown in Fig. 1) revealed full conservation of the introduced mutations (data not shown).

Induction of tumors

About 36-h-old newborns were inoculated with approximately 1 × 10^5 infectious centers of virus and kept under daily inspection until development of disease. Tumors were diagnosed on the basis of gross appearance of lymphoid organs as previously described (Schmidt et al., 1984), combined with cytologic and anatomic criteria (Pattengale, 1994).

DNA extraction and sequence verification of proviruses

Genomic DNA was extracted by DNeasy Tissue kit (Qiagen). The PCR primers used for sequence verification (Fig. 1) will amplify a 369-bp viral fragment that includes all the two-and-a-half 72-bp repeats as well as the UCR sequence. PCR products were analyzed by agarose gel electrophoresis and sequenced on an automated ABI 373 DNA sequencer using the sequencing primer 5’TTGAGACAGTTTCTGGGTCTCTTGAACTGTTGTTG3’.

Southern blotting

Tumor DNAs were digested with HindIII, resolved on a 0.8% agarose gel, blotted onto a membrane, and hybridized with following probes: J1 and J2 (TCR-β chain joining region 1 and 2, respectively), Igκ (immunoglobulin-κ), and Eco (ectropic envelope) as described previously (Amoft et al., 1997).

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