Genetic Determinant of Rapid-Onset B-Cell Lymphoma by Avian Leukosis Virus

MITCHELL R. SMITH,¹ RALPH E. SMITH,² IRA DUNKEL,³ VICTOR HOU,⁴ KAREN L. BEEMON,^{4*}
AND WILLIAM S. HAYWARD³

Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111¹; Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523²; Laboratory of Molecular Genetics and Oncology, Memorial Sloan Kettering Cancer Center, New York, New York 10021³; and Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218⁴

Received 28 February 1997/Accepted 20 May 1997

Infection of 10 day-old chicken embryos with the recombinant avian leukosis virus (ALV) EU-8 induces a high incidence of rapid-onset B-cell lymphoma by insertional activation of the *c-myb* gene. LR-9, a related ALV with differences from EU-8 in the *gag* and *pol* genes, induces rapid-onset lymphoma at only a low incidence. To localize the viral determinant(s) responsible for this biologic difference, we constructed and tested a series of reciprocal chimeras between EU-8 and LR-9 ALVs. The ability to induce rapid-onset lymphoma efficiently was localized to a 925-nucleotide (nt) region of the EU-8 *gag* gene. Sequence analysis of this region revealed a 42-nt deletion in EU-8 relative to LR-9, as well as some single-nucleotide changes. A mutant virus, Δ LR-9, constructed by deleting these 42 nt from LR-9, also induced rapid-onset lymphoma at a high frequency, confirming the biologic significance of this deletion. This deletion removed nt 735 to 776, which lies within a *cis*-acting RNA element that negatively regulates splicing (NRS). The deletion was shown to cause an increase in splicing efficiency, which may lead to increased production of a truncated *myb* gene product from an ALV-*myb* readthrough RNA.

The recombinant avian leukosis virus (ALV) EU-8 induces a high incidence of rapid-onset B-cell lymphoma, causing death as early as 4 to 5 weeks after infection of 10-day-old chicken embryos (38). There is a striking difference in latency between rapid-onset lymphoma and the classic long-latency lymphoma induced by other strains of ALV, which typically cause death 4 to 10 months after infection (reviewed in references 8 and 23). This difference in latency correlates with different sites of proviral integration: the *c-myb* locus in rapid-onset lymphoma (19) and the *c-myc* locus in the long-latency disease (14). More than one oncogene appears to be involved in the long-latency lymphomas; viral activation of *c-bic*, together with *c-myc*, is often observed, particularly in metastatic tumors (9, 43). These two types of B-cell lymphoma are also distinct biologically. The rapid-onset lymphomas become systemic very early after infection, predominantly involving the liver. While the bursa often develops hyperplasia, bursal nodules are not observed (19). However, bursectomized birds do not develop lymphomas after EU-8 ALV infection, suggesting that the bursa is required for the rapid-onset lymphomas (39). In contrast, the long-latency lymphomas demonstrate transformed bursal follicles initially, followed by one, or occasionally more, bursal nodules and finally systemic disease (10, 32).

Different strains of ALV demonstrate significant differences in their ability to induce rapid-onset or long-latency lymphomas (8, 9, 14, 19, 23, 34, 35, 38). The viral genetic determinants of rapid-onset and long-latency lymphomas may reflect the specific requirements for oncogenic activation of the initial target genes, *c-myb* and *c-myc*, respectively. The mechanisms of insertional activation of these two proto-oncogenes differ in several significant ways, as follows. (i) Transcription of the

chimeric ALV-*myb* mRNA initiates in the 5' long terminal repeat (LTR) (18), whereas transcription of ALV-activated *c-myc* almost invariably initiates in the 3' LTR (14). (ii) The *myb*-associated proviruses (with one exception) appear to be structurally intact (19), whereas *myc*-associated proviruses contain internal deletions thought to inactivate transcription from the 5' LTR (5, 13). (iii) Generation of the chimeric ALV-*myb* mRNA requires readthrough of the poly(A) signal in the 3' LTR and splicing from the 5' splice site in *gag* to the 3' splice site in *myb* exon 2, bypassing the *env* 3' splice site (18). Neither of these events is required to generate the 3' LTR-initiated *myc* transcripts. (iv) The rapid-onset lymphomas involve truncation of the Myb protein at its N terminus by 20 amino acids; in contrast, the product of the insertionally activated *c-myc* gene is not truncated. When this minimally truncated *myb* gene product is expressed in a retroviral vector, it induces a high incidence of rapid-onset B-cell lymphomas, as well as sarcomas and adenocarcinomas (18).

EU-8 and LR-9 are related recombinant ALVs that differ only in the *gag-pol* region and yet induce different pathologies (38). Both viruses have the subgroup A *env* gene of UR2-associated virus (UR2AV) and the LTRs of ring-necked pheasant virus (RPV). EU-8 ALV, which contains the RPV *gag* and *pol* genes, induces a high incidence of rapid-onset lymphomas. In contrast, LR-9, which has *gag* and *pol* genes derived from UR2AV, predominantly induces classic long-latency lymphomas (19, 38). These studies suggest that the primary genetic determinant of rapid-onset lymphomas is present in the RPV *gag* or *pol* gene.

To further localize the critical *gag-pol* determinant for rapid-onset lymphomagenesis, we have constructed reciprocal chimeric viruses between EU-8 and LR-9 ALVs and tested their pathogenicities. A fragment of the EU-8 *gag* gene necessary for rapid-onset lymphomas was found to contain a 42-nucleotide (nt) deletion of nt 735 to 776. Deletion of this region, which

* Corresponding author. Mailing address: Department of Biology, Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218. Phone: (410) 516-7289. Fax: (410) 516-7292. E-mail: klb@jhu.edu.

FIG. 1. EU-8/LR-9 recombinant viral plasmids. Recombinant plasmids were generated from the EU-8 and LR-9 parental ALVs by using the restriction sites shown. Black boxes denote EU-8-specific sequences (derived from RPV), which are in the *gag-pol* region (nt 259 to 4999). The positions of the recombination sites for these viruses are shown in Table 1. The oncogenicity of each of these viruses was tested, as shown in Table 1. LR-9 has the LTR of RPV and the rest of the genome of UR2AV. EU-8 has the *env* gene of UR2AV and the LTR and *gag* and *pol* genes of RPV (38).

falls within the matrix (MA)-coding region of the *gag* gene (37) and also within a *cis*-acting RNA element that negatively regulates splicing (NRS) (1, 27), was found to be sufficient for induction of rapid-onset lymphoma. The deletion caused an increase in RNA splicing efficiency and may thus lead to increased production of the spliced ALV-*myb* mRNA in EU-8 lymphomas.

MATERIALS AND METHODS

Construction of recombinant plasmids. Recombinant ALV plasmids were constructed from viral DNAs that were subcloned into the *SacI* site of plasmid pUC12 as previously described (38). Various restriction fragments were exchanged between the plasmid forms of EU-8 and LR-9 ALVs, and the resulting constructs are diagrammed in Fig. 1.

To construct pΔLR-9, with a 42-nt deletion 3' of the *MroI* site in *gag*, PCR primers ΔLR9-S702 (5'-CAGGTCCGGAATGCATCGAGAAACCAGCAACGGCGCAGCGAGATGCGAAGATGG-3') and PrC-AS2750 (5'-GGAACAA GCTTGCGGTAAAC-3') were generated. The ΔLR9-S702 5' primer contains the *MroI* site and pLR9 sequences on either side of the 42-nt deletion, while the PrC AS2750 3' primer was based on the Pr-C RSV sequence (36). PCR was performed on the pLR-9 DNA template by standard techniques with 1.5 mM MgCl₂ and an annealing temperature of 50°C. The product was cloned into pCR2000 (Invitrogen) according to the manufacturer's instructions. The *MroI* fragment of this plasmid was gel purified and ligated to the pLR-9 8-kb *MroI*

fragment. The 2.6-kb *BglII* fragment of pLR-9 was then ligated to the 7.4-kb *BglII* fragment of this intermediate so that only the 925-nt *MroI-BglII* fragment was derived by PCR. DNA sequence analysis of the PCR-generated region confirmed that ΔLR-9 differed from LR-9 only in the 42-nt deletion.

Cloned DNA was sequenced by the dideoxy chain termination method (Sequenase; U.S. Biochemicals) with double-stranded templates. Sequencing primers for the *SacI-BglII* fragment were synthesized with 5' ends at sequences corresponding to RSV nt 520 and 850. All primers were synthesized at the Memorial Sloan Kettering Cancer Center Biochemistry Core Facility.

Chicken embryo fibroblast (CEF) transfection and virus production. C/O *gs⁻chf⁻* fibroblasts from the SC line of White Leghorn chickens (Hyline International, Dallas Center, Iowa) were transfected by the calcium phosphate method with concatameric plasmid DNA as previously described (19, 38). Transfected cells were passaged three times. To collect viral supernatants, confluent plates were fed with fresh complete medium; 12 to 16 h later, medium was collected and frozen. An aliquot was assayed for reverse transcriptase activity, which was related to a standard curve as described previously (38). The titers of the recombinant ALVs and of ΔLR-9 were similar to those of the parental LR-9 and EU-8 viruses.

Inoculation of chickens. Ten-day-old White Leghorn SC chicken embryos were injected with approximately 10⁵ to 10⁶ infectious units of virus via a chorioallantoic membrane vein (38). Chickens were observed daily and sacrificed either when abdominal distension was evident or at 8 or 10 weeks of age. Postmortem examinations were performed on all chickens. Tissues were fixed in 10% buffered Formalin for histopathology or frozen for DNA extraction. Histologic analysis was performed, with the examiner blind to the viral origin of the necropsy materials, as described previously (2).

Splicing assay. DNA fragments containing the NRS (*MroI-SphI*; nt 706 to 1010) were isolated from pLR-9, pEU-8, and pΔLR-9. Each NRS fragment was inserted into the *SacII* site in the *myc* intron of pRSV Neo-int (25). Plasmids containing NRS fragments were transfected into the KBMC cell line with DEAE-dextran as described previously (17). The KBMC line is a reticuloendotheliosis virus strain T-transformed chicken B-cell line (24), which we obtained from Alanna Ruddell, University of Rochester. RNA was isolated 48 h later, and splicing was assayed by RNase protection with an antisense riboprobe spanning the 5' *myc* splice site as described previously (27). Spliced and unspliced RNAs were quantified on an InstantImager (Packard). The previously described *MroI-SphI* (MS) NRS fragment from RSV (27) was tested in parallel with those from EU-8, LR-9 and ΔLR-9. The RSV NRS fragment inserted in the antisense orientation was assayed as a negative control.

RESULTS

Time course study of mortality in ALV-infected chickens.

Our previous study showed that the primary genetic determinant of rapid-onset lymphoma induced by EU-8 ALV was within its *gag-pol* region (38). The goals of this study are to further define the viral genetic determinant(s) in this region, using reciprocal viral constructs, and to provide some insight into the possible mechanism of lymphomagenesis. We initially compared the latent periods of the two parental viruses, EU-8 (*gag-pol* from RPV) and LR-9 (*gag-pol* from UR2AV), in order to determine the optimal time point for a rapid screen of recombinant viruses.

Results of a kinetic study of mortality of chickens infected as 10-day-old embryos with either EU-8 or LR-9 ALV and diagnosed with B-cell lymphoma are shown in Fig. 2. EU-8 ALV infection resulted in a very high incidence of rapid-onset lymphoma, with death occurring from 35 to 145 days after infection and a median time of death of about 70 days. All of the EU-8-induced tumors examined showed proviral integrations in the *myb* locus (18, 19). In contrast, deaths induced by LR-9 were biphasic. A small number (9%) of chickens died 64 to 73 days after infection; these deaths overlapped with some of the deaths in EU-8-infected birds. However, the majority of deaths in LR-9-infected birds (35%) occurred only after a relatively long latent period of 114 days or more. The late tumors induced by LR-9 appeared to be typical long-latency lymphomas, with integrations predominantly at the *myc* locus (three of three examined), whereas both of the two rapid-onset LR-9-induced lymphomas that were analyzed contained proviruses integrated at the *myb* locus. Thus, both EU-8 and LR-9 induce B-cell lymphomas when injected into 10-day-old embryos, but

FIG. 2. Mortality of chickens infected as 10-day-old embryos with EU-8 and LR-9 ALVs. Only mortality of birds diagnosed with B-cell lymphoma is displayed. Deaths are plotted relative to the latent period (days after infection). A total of 49 chickens were infected with EU-8 ALV, and 36 of these were diagnosed with lymphoma. All EU-8-infected birds were dead by 144 days after infection. Other lesions found in EU-8-infected birds included fibrosarcoma (12 birds), lung angiosarcoma (5 birds), fibromatosis (30 birds), and nephroblastoma, hemangioma, and blood cysts (1 bird each). A total of 23 chickens were infected with LR-9 ALV, and 10 of these were diagnosed with lymphoma. By 144 days after infection, 13 of the 23 LR-9-infected birds survived. Other lesions observed in LR-9-infected chicks included fibrosarcoma (2 birds), lung angiosarcoma (1 bird), fibromatosis (1 bird), blood cysts (2 birds), osteopetrosis (12 birds), and anemia (8 birds). Osteopetrosis and anemia occurred mainly at late times after infection (greater than 144 days).

only EU-8 induces a high incidence of the rapid-onset lymphoma involving the *myb* locus.

The data in Fig. 2 suggested that the incidence of lymphoma assayed at 8 to 10 weeks after infection could be used as a specific indicator of rapid-onset lymphoma. Therefore, in subsequent screening of recombinant viruses, all surviving infected chickens were sacrificed 8 weeks after hatching (67 days after infection), and tissues were examined for B-cell lymphoma. At this time point, lymphomas were found in 63% of EU-8-infected birds but in only 13% of LR-9-infected birds (Table 1).

TABLE 1. Incidence of B-cell lymphoma^a

Virus	EU-8-specific sequence (nt) ^b	No. with lymphoma/total (%)
LR-9	None	7/53 (13)
EU-8	259–4999	21/33 (63)
GR	259–2733	6/15 (40)
PR	2733–4999	2/13 (15)
G5'R	259–1630	8/14 (57)
G3'R	1630–2733	0/10 (0)
MS-10	259–706	2/21 (10)
MS-9	706–1630	18/31 (58)
ΔLR-9	Δ735–776	10/25 (40)

^a Viruses are recombinants of LR-9 and EU-8 parental ALVs and are depicted in Fig. 1. Both parental viruses contain the RPV LTR and the UR2AV *env* gene. They differ in the *gag* and *pol* genes: the EU-8-specific sequences in *gag* and *pol* were originally derived from RPV, whereas the LR-9 sequences were from UR2AV. All viruses were injected into 10-day-old chick embryos as described in Materials and Methods. The incidence of B-cell lymphoma was assessed after sacrifice of 8-week-old chicks (67 days after infection).

^b Nucleotide numbering is for Pr-C RSV according to Schwartz et al. (36).

As expected, these levels were somewhat higher than those shown in Fig. 2 at 67 days, because lymphomas in the time course study (Fig. 2) are scored only when they have developed sufficiently to kill the bird.

Critical lymphomagenic determinant localized to the *gag* gene in recombinant ALVs. To localize the determinant in the *gag-pol* region of EU-8 responsible for its high incidence of rapid-onset lymphoma, the series of reciprocal recombinant ALVs shown in Fig. 1 was constructed from EU-8 and LR-9 parental viruses. These recombinants were all derived on an LR-9 background by substituting fragments of the EU-8 *gag* or *pol* genes. Recombinant viral plasmids were transfected into CEFs, and virus was isolated for injection into 10-day-old chicken embryos. The pathology induced by these recombinant viruses was assessed 8 weeks after the chicks hatched (Table 1).

The initial recombinant constructs were designed to assess independently the contributions of the *gag* and *pol* regions of the viral genomes to pathogenesis. The unique *Hpa*I site (Pr-C nt 2733), 230 nt downstream of the beginning of the *pol* coding region, provided a means to separate these two genetic elements (Fig. 1). The virus PR, containing most of the *pol* gene of EU-8, gave a low background incidence of early lymphomas (15%), similar to that of LR-9; the reciprocal construct GR, containing the *gag* gene of EU-8, induced a relatively high incidence of lymphomas (40%) (Table 1).

Next, the *Bgl*II site at Pr-C nt 1630 was used to divide the EU-8 *gag* gene into a 1.4-kb 5' fragment and a 1.1-kb 3' fragment, generating G5'R and G3'R constructs (Fig. 1). The ability to induce rapid-onset lymphomas segregated with G5'R (57% incidence) as opposed to G3'R (0%) (Table 1). This implicated the region including the 5' portion of the EU-8 *gag* gene and upstream leader sequences (nt 259 to 1630) in induction of rapid-onset lymphomas.

Sequence analysis revealed a 42-nt deletion in the region of the *gag* gene necessary for rapid-onset lymphoma. Sequencing of the 1.4-kb *Sac*I-*Bgl*II fragment (nt 259 to 1630) of the two parental plasmids, pEU-8 and pLR-9, was carried out to look for differences that might be important for lymphomagenesis. Two major differences were observed, as well as multiple single-base changes. EU-8 contained a 19-nt insertion generating a repeated sequence (TGCTCTGCGTGATTCCGGT) not present in LR-9, at nt 363 in the noncoding leader region upstream of *gag*. A Rous-associated virus-2 clone contains this identical 19-nt insertion (4). In Pr-C RSV, cDNAs both with and without a similar 17-nt repeat sequence were found at this site (36). In addition, EU-8 contained a 42-nt deletion of nt 735 to 776 (Fig. 3). The deletion is within the *gag* coding region (MA protein domain) (36) but does not alter the translational reading frame. The deletion is also within an RNA element termed the NRS (1, 28), which is inhibitory to splicing. The NRS sequences (nt 700 to 930) of LR-9 and EU-8 ALVs are compared in Fig. 3.

Plasmids pMS-9 and pMS-10 were constructed by using the *Mro*I site (Pr-C nt 706) (Fig. 1) to separate the 19-nt insertion from the 42-nt deletion in G5'R. The results of infection with MS-9 and MS-10 viruses demonstrated that the rapid lymphomagenic potential was carried by the *Mro*I-*Bgl*II segment (nt 706 to 1630) from EU-8. MS-9 was nearly as active in inducing rapid-onset lymphomas (58%) as was EU-8 (63%) (Table 1), yet it differed from LR-9 by only the 42-nt deletion and the scattered single-base changes. In contrast, the reciprocal construct MS-10 (nt 259 to 706 from EU-8) induced lymphomas in only 10% of the infected birds (Table 1).

Lymphomagenic determinant localized to 42-nt deletion in *gag*. To test whether the critical determinant was the 42-nt deletion in *gag*, we constructed ΔLR-9, which differs from LR-9

FIG. 3. Sequences of the NRSs of LR-9 and EU-8 ALVs. The sequences of the NRS elements (nt 700 to 930) from LR-9 and EU-8 are compared. The asterisks denote deleted nucleotides in the EU-8 sequence. The LR-9 sequence is very similar to that of Pr-C RSV (36) but with a few scattered nucleotide changes.

only in the 42-nt deletion. Δ LR-9 induced lymphomas in 40% of the birds by 8 weeks of age, whereas LR-9 induced lymphomas in only 13% (Table 1). This observation was confirmed in a second experiment in which the time course of deaths due to lymphoma in Δ LR-9-infected birds was monitored. Deaths occurred as early as 40 days after infection and reached 54% by day 81 (Fig. 4). Overall, the kinetics of mortality from lymphomas for Δ LR-9 (Fig. 4) closely paralleled those for EU-8 (Fig. 2) and differed markedly from those for LR-9 (Fig. 2 and 4). Thus, this 42-nt *gag* deletion in EU-8 is sufficient to convert LR-9 from a virus primarily producing late-onset lymphomas to one efficiently inducing rapid-onset lymphomas. Analysis of proviral integration sites in representative Δ LR-9-induced lymphomas showed them to involve integrations in the *myb* locus (three of three).

Incidence of other proliferative diseases. While not directly relevant to the goals of this study, other lesions were observed in birds infected with certain of these recombinant viral constructs. Proliferative fibromatosis, a nonmalignant lesion previously shown to be associated with infection by certain ALV strains (22, 38), was diagnosed in some birds and appeared to correlate roughly with the 42-nt *gag* deletion in the constructs described above (data not shown). Nothing is known about the

molecular basis of this disease, and this observation was not pursued further.

Early proliferative vascular lesions in the lung that histologically appear to be angiosarcomas are induced at a high incidence (up to 80%) by subgroup F ALV (7, 38). A low incidence of this lesion (0 to 29%) was observed with the present series of subgroup A constructs (Fig. 1), but no consistent pattern implicating a specific genetic determinant emerged.

***gag* deletion increases splicing efficiency.** The 42-nt deletion in the *gag* gene of Δ LR-9 was shown above to be sufficient for efficient induction of rapid-onset lymphomas (Fig. 4), raising the question of the functional role of this deletion in lymphomagenesis. This in-frame deletion falls within the C-terminal region of the *gag* matrix protein (MA), deleting amino acids 119 to 132 of this 155-amino-acid polypeptide. The C-terminal portion of MA, including the region deleted in Δ LR-9, has been shown to be dispensable for viral infectivity and budding (33).

This 42-nt deletion also falls within the NRS RNA element (Fig. 3), previously identified in avian retroviruses at nt 700 to 930 (1, 27). This NRS element, together with suboptimal 3' splice sites (20, 21, 28), is involved in limiting splicing of avian retroviruses in order to maintain full-length viral RNA for use as *gag-pol* mRNA and for packaging into virions. Since the rapid-onset lymphomas involve splicing of readthrough transcripts from the viral 5' splice site to the 3' splice site of *myb* exon 2 (18), it seemed possible that the deletion might be affecting the amount of spliced ALV-*myb* RNA generated by altering the splicing efficiency of the readthrough transcripts.

To test this possibility, DNA fragments containing the NRS sequence from the Pr-C strain of RSV, LR-9, EU-8, and Δ LR-9, were inserted into the chicken cellular *myc* intron 2 in an NRS test construct previously used by McNally et al. (27). Plasmids were transiently transfected into the KBMC chicken B-cell line (24), and steady-state levels of spliced and unspliced RNAs were measured by an RNase protection assay, using a probe spanning the 5' splice site. In a representative experiment shown in Fig. 5, the NRS from LR-9, like the RSV NRS, inhibited splicing efficiently. In comparison, the control construct containing the RSV NRS in the antisense orientation, in which it is inactive (27), yielded predominantly spliced RNA.

Insertion of the Δ LR-9 NRS sequence into this same test construct resulted in an intermediate level of spliced RNA; in six different experiments, this represented at least a twofold increase in spliced RNA levels over that observed with the wild-type LR-9 NRS construct (Fig. 5). Results with the EU-8 NRS construct were very similar to those with the Δ LR-9 construct (Fig. 5). This suggested that the additional single-

FIG. 4. Mortality due to B-cell lymphoma in chickens infected with LR-9 or Δ LR-9 as 10-day-old embryos.

FIG. 5. Comparison of NRS activities of LR-9, EU-8, and Δ LR-9. The NRS fragments (nt 706 to 1010) from the viruses shown were inserted into the Neo-int test plasmid (25). Splicing was assayed by transient transfection of KBMC chicken B cells, followed by an RNase protection assay. The riboprobe spans the 5' splice site as shown. The RSV NRS was inserted in both sense (+) and antisense (-) orientations and served as a positive and negative control, respectively. SV40, simian virus 40.

base changes between the EU-8 and Δ LR-9 NRS sequences did not affect NRS activity significantly and that the decrease in splicing inhibition was a result of the 42-nt deletion. These data support our hypothesis that the deletion may facilitate splicing of ALV-*myb* readthrough pre-mRNAs, thus generating higher levels of the chimeric mRNA that encodes the truncated Myb protein (18).

DISCUSSION

A genetic determinant within the ALV *gag* gene is important for the induction of rapid-onset lymphomas in chickens infected as 10-day-old embryos. The data presented here show that a 42-nt deletion in the EU-8 *gag* gene is the *gag-pol* determinant most important in conferring this pathogenicity. We show here that introduction of this deletion into the LR-9 *gag* gene is sufficient to convert this low-incidence strain of ALV into a virus inducing a high incidence of rapid-onset lymphomas with *myb* integrations.

The deletion is in frame and removes amino acids 119 to 132 from the C-terminal portion of MA encoded by the ALV *gag* gene; however, this deletion does not appear to block viral replication. Similarly, Nelle and Wills (33) showed that deletion of MA amino acids 114 to 142 from RSV did not abolish viral infectivity, although budding efficiency appeared to be decreased. Thus, we cannot exclude the possibility that viruses harboring this deletion would spread more slowly in the infected birds. If this is true, however, the greatly enhanced incidence of rapid-onset lymphoma associated with the deletion would be all the more remarkable.

A *cis*-acting negative regulator of splicing (NRS) is located at approximately nt 700 to 930 in the *gag* genes of all avian retroviruses (1, 27). This RNA element is involved in control of retroviral splicing to generate full-length genomic RNA and mRNA as well as spliced mRNA; Gag and Pol proteins are translated from full-length mRNAs, and Env proteins are translated from spliced mRNA. Partial deletion of the NRS, as in EU-8 and Δ LR-9 (Fig. 3), partially abrogated NRS activity and increased the amount of spliced RNA in our test system (Fig. 5). We hypothesize that this could lead to increased

splicing of readthrough RNA to downstream cellular genes, such as the *c-myc* coding sequences located downstream of viral integration sites in rapid-onset lymphomas (18).

The ALV-*myb* readthrough transcripts contain a 5' splice site in *gag* and two alternative 3' splice sites downstream: *env* and *c-myc* exon 2. There is likely to be competition between these two splice sites, much as occurs in RSV, where the alternative 3' splice sites are *env* and *src*. Deletion of the NRS sequence from RSV constructs leads to increased splicing from the viral 5' splice site to both suboptimal 3' splice sites (42). However, in ALV-*myb* pre-mRNA splicing, the constitutive splice site of *c-myc* exon 2 would presumably be preferred over the suboptimal 3' splice site of *env* (11, 20, 21). ALV-*myb* pre-mRNA splicing also differs from that of RSV, since *c-myc* has 15 exons (40) whereas RSV has only 2 exons, (*env* and *src* are alternative 3'-terminal exons). Thus, the NRS is located upstream of an internal rather than a terminal exon in the ALV-*myb* readthrough pre-mRNA. Size constraints have been observed to affect internal exons, flanked by large introns, such that internal exons larger than approximately 500 nt are not efficiently spliced (3, 41); terminal exons are not subject to the same size constraints. When readthrough occurs through the 3' LTR, the *env* exon is converted from an approximately 2-kb terminal exon to an internal exon ranging from over 2 kb up to 8 kb (depending on integration site), further suggesting that *c-myc* would be preferred over *env* as the 3' splice site.

The NRS binds a number of splicing factors, including U1, U2, and U11/U12 small nuclear ribonucleoproteins (12) and SR proteins (29). The 42-nt deletion in EU-8 and Δ LR-9 is within a purine-rich portion of the NRS that has been shown recently to bind ASF/SF2 and other SR proteins (29), which are important for both constitutive and alternative splicing (6, 26, 44). Thus, it is possible that the deletion affects binding of these SR proteins proposed to be necessary for full NRS activity. Since the NRS has previously been shown to affect the splicing only of the intron that contains it (27), we would not expect the NRS to affect splicing of the remaining *myb* exons.

Comparison of the EU-8 ALV lymphoma system with murine leukemia virus (MuLV) induction of myeloid leukemia in mice shows both intriguing similarities and differences. Both viruses integrate into the *c-myc* gene in tumors, and a virus-*myb* readthrough transcript is spliced from a viral 5' splice site to a *myb* 3' splice site in both cases (18, 19, 37). However, the resulting *myb* product is generally more truncated in the mouse leukemia due to viral integration sites that are further downstream (37). Study of recombinant MuLVs showed that induction of leukemia requires determinants present in the *gag* gene of Moloney MuLV but not in Friend MuLV (31). In contrast to ALV, where the *gag* determinant was localized to a 42-nt deletion, the MuLV determinants were localized to a much larger region, requiring both the psi-*gag*-PR and *env* sequences of Moloney MuLV (31). Further, a cryptic 5' splice site within the MuLV *gag* gene is used in the MuLV-induced mouse myeloid leukemia (37), whereas the normal 5' splice site in the ALV *gag* gene is used in the rapid-onset chicken B-cell lymphomas studied (18). Another difference between these two systems is that Moloney MuLV is a naturally occurring virus, while EU-8 is a recombinant. In addition to the critical *gag* determinant, an ALV with the RPV LTR and a subgroup A *env* gene is required for a high incidence of rapid-onset lymphoma (38).

ALV-induced erythroblastosis also involves ALV readthrough transcripts; these are spliced from ALV sequences into the *c-erbB* gene, producing an N-terminally truncated ErbB protein (23). However, the splicing pattern in erythroblastosis is more complex than that in EU-8 lymphomas, using a cryptic 5'

splice site in *env* and a 3' splice site in *c-erbB*, as well as the normal splice sites in *gag* and *env* (23).

Another regulatory function localized to the ALV *gag* gene is promotion of polyadenylation of viral RNA sequences in the 3' LTR. The polyadenylation and cleavage signals in ALV LTRs are relatively inefficient; approximately 15% of transcripts typically read past these signals in CEFs (15, 30). Deletion of the *gag* region from nt 630 to 1149 leads to a threefold increase in this readthrough RNA (30). Since the deletion in Δ LR-9 falls within this region of *gag*, it was possible that it could increase readthrough transcripts, which might in turn increase the likelihood of activation of a downstream cellular gene, e.g., *c-myc*. However, LR-9 and Δ LR-9 appear to have similar readthrough levels in CEFs and chicken B cells, suggesting that the 42-nt deletion is probably not involved in readthrough control (16).

In summary, a 42-nt deletion was identified within a *cis*-acting inhibitor of splicing (the NRS) in the *gag* gene of EU-8. Creation of this deletion in LR-9 converts it from a virus that induces primarily long-latency *c-myc* lymphomas to a virus that induces a high incidence of rapid-onset lymphomas involving *c-myc*. The mechanisms by which *c-myc* and *c-myb* are insertionally activated differ in a number of ways, including the manner in which the respective functional mRNAs are generated. Transcription of the ALV-*myc* RNA in long-latency lymphomas initiates in the viral 3' LTR and reads directly into adjacent *myc* sequences; viral splice sites are not involved in generating the functional mRNA (8, 23). By contrast, the ALV-*myb* RNA in rapid-onset lymphomas initiates in the viral 5' LTR and continues through the 3' LTR into downstream *myb* sequences; the functional mRNA is generated by splicing from the viral 5' splice site in *gag* to the 3' splice site of *c-myc* exon 2, thus removing downstream viral sequences and any intervening cellular sequences (18). The significance of this process is twofold: not only is *c-myc* placed under the transcriptional control of the viral LTR, but, perhaps even more important, the resulting mRNA encodes a minimally truncated Myb protein with enhanced oncogenic properties (18). We have proposed that the 42-nt deletion of EU-8, by partially inactivating the NRS, enhances splicing from the viral *gag* 5' splice site to 3' splice sites in downstream cellular sequences. This would not be important for *c-myc* activation in long-latency lymphomas, since viral splice sites are not involved, but could have a profound effect on the ability of the virus to generate the spliced ALV-*myb* transcripts associated with *c-myc* activation and hence on its ability to induce rapid-onset lymphomas.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CA43250 and CA16999 (to W.S.H.), CA35984 (to R.E.S.), and CA48746 (to K.L.B.) and by the Revson Foundation (M.R.S.).

We thank Jofrid Torgersen, MaryAnne Carroll, Kenya Parks, Christine McDaniel, and Marguerite Ro for excellent technical assistance and Tsvetelina Pentcheva for review of the manuscript.

REFERENCES

- Arrigo, S., and K. Beemon. 1988. Regulation of Rous sarcoma virus RNA splicing and stability. *Mol. Cell. Biol.* **8**:4858-4867.
- Aurigemma, R. E., J. L. Torgersen, and R. E. Smith. 1991. Sequences from myeloblastosis-associated virus MAV-2(0) and UR2AV involved in the formation of plaques and the induction of osteopetrosis, anemia, and ataxia. *J. Virol.* **65**:23-30.
- Berget, S. M. 1995. Exon recognition in vertebrate splicing. *J. Biol. Chem.* **270**:2411-2414.
- Bizub, D., R. A. Katz, and A. M. Skalka. 1984. Nucleotide sequence of noncoding regions in Rous-associated virus-2: comparisons delineate conserved regions important in replication and oncogenesis. *J. Virol.* **49**:558-565.
- Boerkoel, C. F., and H.-J. Kung. 1992. Transcriptional interaction between retroviral long terminal repeats (LTRs): mechanism of 5' LTR suppression and 3' LTR promoter activation of *c-myc* in avian B-cell lymphomas. *J. Virol.* **66**:4814-4823.
- Caceres, J. F., S. Stamm, D. M. Helfman, and A. R. Krainer. 1994. Regulation of alternative splicing in vivo by overexpression of antagonistic splicing factors. *Science* **265**:1706-1709.
- Carter, J. K., S. J. Proctor, and R. E. Smith. 1983. Induction of angiosarcomas by ring-necked pheasant virus. *Infect. Immun.* **40**:310-319.
- Clurman, B. E., and W. S. Hayward. 1988. Insertional activation of proto-oncogenes previously identified as viral oncogenes, p. 55-94. *In* G. Klein (ed.), *Cellular oncogene activation*. Marcell Dekker, Inc., New York, N.Y.
- Clurman, B. E., and W. S. Hayward. 1989. Multiple proto-oncogene activations in avian leukemia virus-induced lymphomas: evidence for stage-specific events. *Mol. Cell. Biol.* **9**:2657-2664.
- Cooper, M. D., L. E. Payne, P. B. Dent, B. P. Burmester, and R. A. Good. 1968. Pathogenesis of avian lymphoid leukemia. I. Histogenesis. *J. Natl. Cancer Inst.* **41**:374-389.
- Fu, X.-D., R. A. Katz, A. M. Skalka, and T. Maniatis. 1991. The role of branchpoint and 3' exon sequences in the control of balanced splicing of avian retrovirus RNA. *Genes Dev.* **5**:211-220.
- Gontarek, R. R., M. T. McNally, and K. Beemon. 1993. Mutation of an RSV intronic element that abolishes both U₁₁/U₁₂ snRNP binding and negative regulation of splicing. *Genes Dev.* **7**:1926-1936.
- Goodenow, M. M., and W. S. Hayward. 1987. 5' long terminal repeats of *myc*-associated proviruses appear structurally intact but are functionally impaired in tumors induced by avian leukemia viruses. *J. Virol.* **61**:2489-2498.
- Hayward, W. S., B. G. Neel, and S. M. Astrin. 1981. Activation of a cellular *onc* gene by promoter insertion in ALV-induced lymphoid leukemia. *Nature* **290**:475-480.
- Herman, S. A., and J. M. Coffin. 1986. Differential transcription from the long terminal repeats of integrated avian leukemia virus DNA. *J. Virol.* **60**:497-505.
- Hou, V., and K. L. Beemon. 1996. Unpublished results.
- Houtz, E. K., and K. F. Conklin. 1996. Identification of EFIV, a stable factor present in many avian cell types that transactivates sequences in the 5' portion of the Rous sarcoma virus long terminal repeat enhancer. *J. Virol.* **70**:393-401.
- Jiang, W., M. R. Kanter, I. Dunkel, R. G. Ramsay, K. L. Beemon, and W. S. Hayward. 1997. Minimal truncation of the *c-myc* gene product in rapid-onset B-cell lymphoma. *J. Virol.* **71**:6526-6533.
- Kanter, M. R., R. E. Smith, and W. S. Hayward. 1988. Rapid induction of B-cell lymphomas: insertional activation of *c-myc* by avian leukemia virus. *J. Virol.* **62**:1423-1432.
- Katz, R. A., and A. M. Skalka. 1990. Control of retroviral RNA splicing through maintenance of suboptimal processing signals. *Mol. Cell. Biol.* **10**:696-704.
- Katz, R. A., M. Kotler, and A. M. Skalka. 1988. *cis*-acting intron mutations that affect the efficiency of avian retroviral RNA splicing: implication for mechanisms of control. *J. Virol.* **62**:2686-2695.
- Kogekar, N., T. L. Spurgeon, M. C. Simon, and R. E. Smith. 1987. Proliferative fibromatosis in avian skeletal muscle caused by cloned recombinant avian leukemia viruses. *Cancer Res.* **47**:2083-2091.
- Kung, H.-J., C. Boerkoel, and T. H. Carter. 1991. Retroviral mutagenesis of cellular oncogenes: a review with insights into mechanisms of insertional activation. *Curr. Top. Microbiol. Immunol.* **171**:1-225.
- Lewis, R. B., J. McClure, B. Rup, D. W. Niesel, R. F. Garry, J. D. Hoelzer, K. Nazerian, and H. R. Bose, Jr. 1981. Avian reticuloendotheliosis virus: identification of the hematopoietic target cell for transformation. *Cell* **25**:421-431.
- Linial, M. 1987. Creation of a processed pseudogene by retroviral infection. *Cell* **49**:93-102.
- Manley, J. L., and R. Tacke. 1996. SR proteins and splicing control. *Genes Dev.* **10**:1569-1579.
- McNally, M. T., R. R. Gontarek, and K. Beemon. 1991. Characterization of Rous sarcoma virus intronic sequences that negatively regulate splicing. *Virology* **185**:99-108.
- McNally, M. T., and K. Beemon. 1992. Intronic sequences and 3' splice sites control Rous sarcoma virus RNA splicing. *J. Virol.* **66**:6-11.
- McNally, L. M., and M. T. McNally. 1996. SR protein splicing factors interact with the Rous sarcoma virus negative regulator of splicing element. *J. Virol.* **70**:1163-1172.
- Miller, J. T., and C. M. Stoltzfus. 1992. Two distinct upstream regions containing *cis*-acting signals regulating splicing facilitate 3'-end processing of avian sarcoma virus RNA. *J. Virol.* **66**:4242-4251.
- Mukhopadhyaya, R., J. Richardson, V. Nazarov, A. Corbin, R. Koller, M. Sitbon, and L. Wolff. 1994. Different abilities of Friend murine leukemia virus (MuLV) and Moloney MuLV to induce promonocytic leukemia are due to determinants in both *psi-gag-PR* and *env* regions. *J. Virol.* **68**:5100-5107.
- Neiman, P. E., L. Jordan, R. A. Weiss, and L. N. Payne. 1980. Malignant lymphoma of the bursa of Fabricius and analysis of early transformation.

- Cold Spring Harbor Symp. Quant. Biol. 7:519–528.
33. **Nelle, T. D., and J. W. Wills.** 1996. A large region within the Rous sarcoma virus matrix protein is dispensable for budding and infectivity. *J. Virol.* **70**:2269–2276.
 34. **Pizer, E., and E. H. Humphries.** 1989. RAV-1 insertional mutagenesis: disruption of the *c-myb* locus and development of avian B-cell lymphomas. *J. Virol.* **63**:1630–1640.
 35. **Pizer, E. S., T. W. Baba, and E. H. Humphries.** 1992. Activation of the *c-myb* locus is insufficient for the rapid induction of disseminated avian B-cell lymphoma. *J. Virol.* **66**:512–523.
 36. **Schwartz, D. E., R. Tizard, and W. Gilbert.** 1983. Nucleotide sequence of Rous sarcoma virus. *Cell* **32**:853–869.
 37. **Shen-Ong, G. L. C., and L. Wolff.** 1987. Moloney murine leukemia virus-induced myeloid tumors in adult BALB/c mice: requirement of *c-myb* activation but lack of *v-abl* involvement. *J. Virol.* **61**:3721–3725.
 38. **Simon, M. C., W. S. Neckameyer, W. S. Hayward, and R. E. Smith.** 1987. Genetic determinants of neoplastic diseases induced by a subgroup F avian leukosis virus. *J. Virol.* **61**:1203–1212.
 39. **Smith, R. E.** Unpublished results.
 40. **Soret, J., M. Vellard, E. Viegas-Pequignot, F. Apiou, B. Dutrill, and B. Perbal.** 1990. Chromosomal reallocation of the chicken *c-myb* locus and organization of 3'-proximal coding exons. *FEBS Lett.* **263**:254–260.
 41. **Sterner, D. A., T. Carlo, and S. M. Berget.** 1996. Architectural limits on split genes. *Proc. Natl. Acad. Sci. USA* **93**:15081–15085.
 42. **Stoltzfus, C. M., and S. J. Fogarty.** 1989. Multiple regions in the Rous sarcoma virus *src* gene intron act in *cis* to affect the accumulation of unspliced RNA. *J. Virol.* **63**:1669–1676.
 43. **Tam, W., D. Ben-Yehuda, and W. S. Hayward.** 1997. *bic*, a novel gene activated by proviral insertions in avian leukosis virus-induced lymphomas, is likely to function through its noncoding RNA. *Mol. Cell. Biol.* **17**:1490–1502.
 44. **Zahler, A. M., W. S. Lane, J. A. Stolk, and M. B. Roth.** 1992. SR proteins: a conserved family of pre-mRNA splicing factors. *Genes Dev.* **6**:837–847.