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MINIREVIEW

Transcription Regulatory Elements of the Avian Retroviral Long Terminal Repeat

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The U3 long terminal repeat (LTR) region of the avian retroviruses has been extensively characterized as a model of a strong transcription regulatory unit. This compact enhancer and promoter drives high levels of viral and cellular gene transcription in many cell types in birds and in mammals. Viral mRNA and genomic RNA transcripts can comprise up to 20% of the total RNA in infected cells (Varmus and Swanstrom, 1984). The high level expression of the retroviral LTR has been exploited in the development of reporter gene expression vectors (Gorman *et al.*, 1982) and of expression vectors for mammalian gene therapy studies (Yoshimura *et al.*, 1992). Infectious avian retroviral constructs have also been engineered for gene expression studies (Garber *et al.*, 1991; Hughes *et al.*, 1987) and for development of transgenic chickens (Salter *et al.*, 1987). Analysis of this potent transcription regulatory unit will provide insight to the molecular basis for enhancer and promoter function and will help in the design of improved retroviral vectors.

The avian retroviral LTR is also of interest because of its essential role in oncogenesis. The Rous sarcoma virus carries a transduced *v-src* oncogene and rapidly induces fibrosarcoma in chickens, caused by deregulated LTR-driven *v-src* expression (Bishop and Varmus, 1984). Related strains of viruses carry other oncogenes such as *v-fps* or *v-ros*. The slowly transforming avian leukosis viruses (ALV) also known as Rous-associated virus (RAV) strains do not carry a viral oncogene, but instead induce tumors following integration of proviral sequences next to cellular protooncogenes, which gives high levels of LTR-driven oncogene transcription. These integrated proviruses often show large deletions of viral sequences, although the LTR is preserved to drive high level viral and cellular oncogene transcription (Goodenow and Hayward, 1987; Linial and Groudine, 1985). The most common ALV tumors observed are bursal lymphomas which show integration next to the *c-myc* protooncogene (Neel *et al.*, 1981; Payne *et al.*, 1981) and in some cases next to *c-myb* (Kanter *et al.*, 1988). ALV induction of B cell lymphoma may be regulated by LTR enhancer binding proteins that are labile (decreased after protein synthesis inhibition) in the target pre-B cells of ALV-susceptible strains (Ruddell *et al.*, 1988). Developmental regulation of LTR transcription by these labile factors could influence *c-myc* hyperexpression in a manner essential for tumor induction in the bursa (Bowers *et al.*, 1994).

Further evidence that the LTR enhancer is important for retroviral oncogenesis comes from studies of the endogenous avian retroviruses. These viruses are related to the ALV and RSV strains, although they show a number of deletions in the enhancer and promoter region of the LTR (Cullen *et al.*, 1983). The ability of the endogenous viruses to induce tumors in birds is greatly reduced, probably due to the weak transcription activity of the LTR (Cullen *et al.*, 1985a; Motta *et al.*, 1985). Replacement of the endogenous virus U3 LTR region with that of ALV increases the oncogenic activity of the virus (Robinson *et al.*, 1982). These and other experiments suggest that the LTR and its associated transcription activity is essential in determining oncogenic potential. A number of studies have led to a fairly complete picture of the LTR sequences and binding proteins involved in retroviral transcription and tumorigenesis, as discussed in this review.

IDENTIFICATION OF LTR ENHANCER AND PROMOTER ELEMENTS

The RSV LTR was one of the first regulatory elements sequenced and analyzed for transcription activity, using transient transfection assays in tissue culture cells (Gorman *et al.*, 1982; Mitsialis *et al.*, 1983). The enhancer region has been mapped to the 110-bp region positioned at roughly −250 to −140 bp with respect to the transcription start site at the U3-R LTR boundary (Cullen *et al.*, 1985b). This corresponds to the region 5′ of the *Sph*I site shown in Fig. 1. The enhancer can function in either orientation 5′ or 3′ of genes, although the highest levels of transcription enhancement are obtained in the natural configuration 5′ of the gene (Norton and Coffin, 1987; Gowda *et al.*, 1988). The promoter region maps from

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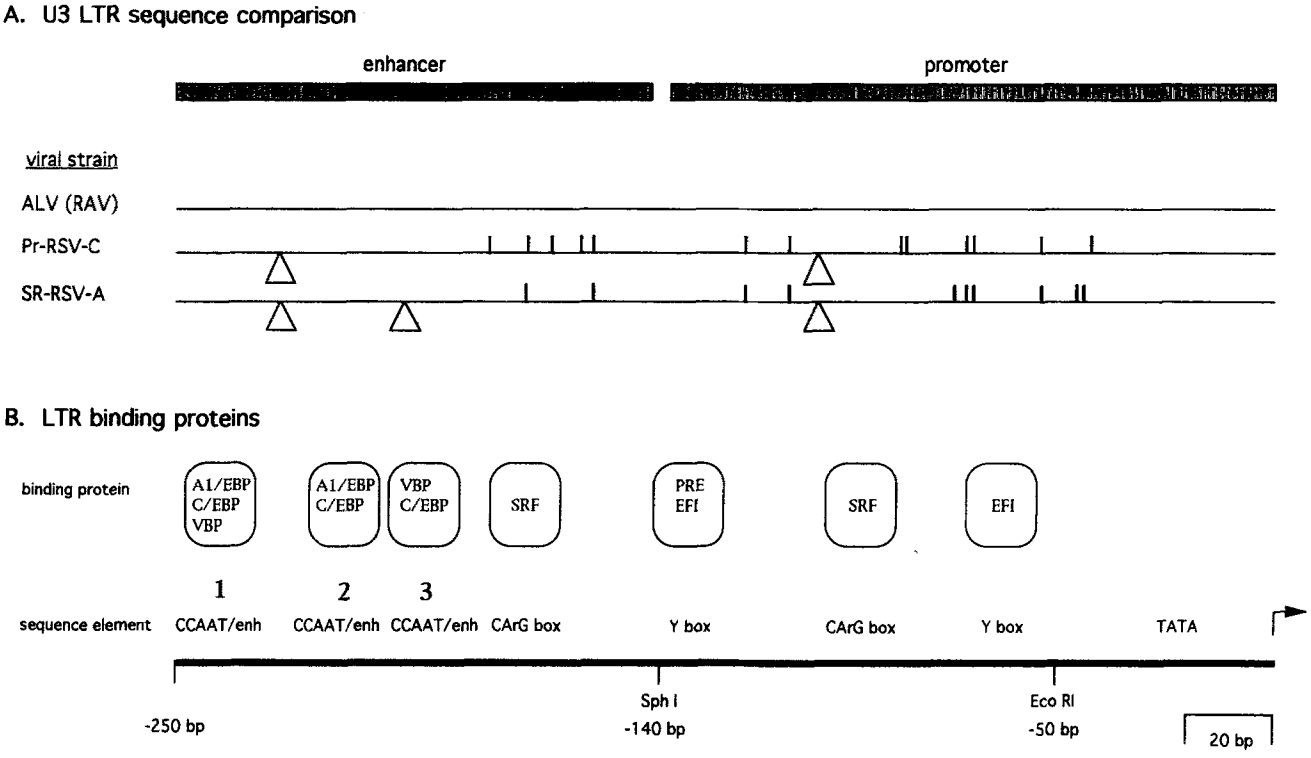

FIG. 1. Map of avian retroviral LTR binding proteins. (A) The U3 sequence of an ALV LTR (BK25 RAV-2 strain) is compared with that of the PR-C and SR-A strain RSV LTRs. Triangles indicate deletions, and notches indicate point mutations. (B) LTR protein binding sequence elements and representative binding proteins. The CCAAT/enhancer (CCAAT/enh) elements are numbered 1 to 3. Restriction sites and the distance from the transcription start site at the U3-R border are indicated.

roughly −140 bp to the transcription start site, including a TATA box at position −24 bp (Cullen *et al.*, 1985b).

The ALV and RSV strain LTRs show a number of sequence differences that are summarized in Fig. 1A. The sequence of a typical ALV LTR that is nearly identical to the RAV-2 strain LTR (Schubach and Horvath, 1988) is compared with that of the Pr-RSV-C and SR-RSV-A strains (Bizub *et al.*, 1984). The RSV strains show a few small deletions (5−14 bp) and several point mutations relative to the ALV LTR sequence (Fig. 1A). The same sequence variations were observed in more comprehensive comparisons of the ALV and RSV LTRs of different strains (Majors, 1990; Bizub *et al.*, 1984). All of these strains show high levels of viral transcription, suggesting that the sequence differences do not appreciably influence LTR function.

CHARACTERIZATION OF LTR BINDING PROTEINS

The U3 LTR region has been intensively screened to identify the binding factors involved in transcription regulation. The major protein binding motifs identified include CCAAT/enhancer, CArG box, and Y box elements. A summary of the ALV and RSV LTR protein binding sites is given in Fig. 1B, with minor differences in protein binding by each viral LTR that are discussed below. The 5′ LTR enhancer region contains multiple direct repeats of closely related CCAAT/enhancer elements (from about −250 to −190 bp), showing four in the case of Pr-RSV-C, three in ALV, and two in the SR-RSV-A LTR (Ryden *et al.*, 1993; Smith *et al.*, 1994). Three related motifs are also found in an internal enhancer found in the *gag* gene region of RSV (Karnitz *et al.*, 1987; Ryden *et al.*, 1993). Nuclear extracts from several avian cell types give a complex gel shift pattern with CCAAT/enhancer element probes, and the binding activities have been designated a1 and a3 (Ruddell *et al.*, 1989), EFII (Sealy and Chalkley, 1987), E2BP (Kenny and Guntaka, 1990), or FI and FIII (Goodwin, 1988), as summarized in Table 1. These multiple gel shift binding activities could reflect the contributions of several binding proteins, or could represent protein multimerization (Sears and Sealy, 1994; Smith *et al.*, 1994).

The genes encoding LTR CCAAT/enhancer element binding proteins have been cloned from mammalian cells (reviewed by Johnson and McKnight, 1989). These genes belong to the bZIP family of transcription factors, which share conserved carboxy terminal basic region DNA binding and leucine zipper dimerization motifs, while their amino terminal *trans*-activating domains are variable. The prototype bZIP factor C/EBPα binds the CCAAT/enhancer elements of several cellular gene enhancers as well as the RSV LTR enhancer (consensus $T^{T}/_{G}NNG^{C}/_{T}AA^{T}/_{G}$; where N is any nucleotide; Ryden and Beemon, 1989). Additional bZIP proteins that bind RSV LTR CCAAT/enhancer elements include NF/IL6 (Akira *et al.*, 1990) and Ig/EBP (Roman *et al.*, 1990).

The genes encoding two avian bZIP factors (A1/EBP and VBP) have been cloned from ALV-induced bursal lymphoma cells (Bowers and Ruddell, 1992; Smith *et al.,* 1994). A1/EBP binds overlapping but distinct subsets of LTR CCAAT/enhancer elements when compared with VBP (Smith *et al.,* 1994). A1/EBP binds the consensus sequence $TN^{A}/_{T}TGCAAN$ in CCAAT/enhancer elements 1 and 2, while VBP binds $TT^{G}/_{A}CATAAG$ in sites 1 and 3 (Fig. 1B). These factors and the other members of the bZIP family could be responsible for the complex gel shift pattern of LTR CCAAT/enhancer binding activity observed in many cell types and species. At least some members of this family can form heterodimers (Vinson *et al.,* 1993), further adding to the potential complexity of binding to LTR CCAAT/enhancer elements. These bZIP factors may encode the labile LTR binding factors thought to be important for ALV tumor induction in B cells (Bowers *et al.,* 1994).

The second major LTR protein binding motif is a CArG box element (Boulden and Sealy, 1990). This sequence element ($CC(^{A}/_{T})_{6}GG$) is found in two sites in the LTR enhancer and promoter (Zachow and Conklin, 1992), at about −170 and −95 bp from the transcription start site (Fig. 1B). The CArG box included in the *c-fos* promoter is the best characterized of this family of elements. The mammalian *c-fos* serum response element binds multiple proteins that appear to regulate serum induction of *c-fos* promoter activity, including serum response factor (SRF; Treisman, 1987) p62/DBP (Ryan *et al.,* 1989) and p62/TCF (Shaw *et al.,* 1989). These proteins could also mediate the roughly fivefold increase in RSV LTR-driven transcription observed in fibroblasts after serum treatment (Boulden and Sealy, 1990; Dutta *et al.,* 1990). An avian serum response factor (EFIII) has been identified which binds the CArG boxes in the RSV LTR and in the *c-fos* promoter (Boulden and Sealy, 1992). The EFIII activity is recognized by a *Xenopus* SRF antiserum, suggesting that it encodes the avian SRF homolog. A second avian factor (SREBP) efficiently binds sequences 3′ of the serum response element in the *c-fos* promoter, but shows weak affinity for the RSV LTR CArG boxes. Proteins binding the RSV LTR CArG boxes show increased gel shift binding activity after serum treatment of rat fibroblasts, which could contribute to the serum responsiveness of RSV LTR-driven transcription in mammalian cells (Lang *et al.,* 1993).

The third major LTR protein binding motif is a Y box or inverted CCAAT box motif (GGTTA) present in two promoter sites (at −140 and −65 bp) in the ALV and RSV LTRs (Fig. 1B). Proteins binding to these LTR elements have been identified in a variety of avian cell types (EFI, Sealy and Chalkley, 1987; FII, Goodwin, 1988; C, Ruddell *et al.,* 1988) and in mammalian cells (CBF, Hatomochi *et al.,* 1988), as summarized in Table 1. The Y box binding activity contains multiple components which have been separated by biochemical methods (Hatomochi *et al.,* 1988; Faber and Sealy, 1990). The genes encoding Y box LTR binding proteins have been cloned from many species including chickens (YB-1, Grant and Deeley, 1993; EFI, Kandala and Guntaka, 1994), rats (CBF-A, Vuorio *et al.,* 1990; CBF-B, Maity *et al.,* 1990; EFIa, Ozer *et al.,* 1990), and mice (NF-YA and NF-YB; Hooft van Huijsduijnen *et al.,* 1990). These genes represent two types of transcription factors. The YB-1/EFI factors are nearly identical, and they show relatively nonspecific binding to pyrimidine-rich double- and single-stranded DNA sequences such as the ATTGG motif (Grant and Deeley, 1993). The CBF/NF-Y factors are related to the heterodimeric HAP2 and HAP3 yeast proteins (Hooft van Huijsduijnen *et al.,* 1990; Maity *et al.,* 1990; Vuorio *et al.,* 1990). The proposed DNA binding region of these proteins is highly conserved and very different than that of other transcription factors (Li *et al.,* 1992). It is not yet known whether some or all of these factors contribute to the Y box LTR binding activity observed in nuclear extracts.

A fourth LTR binding activity has been identified in the region just 5′ of the *Sph*I site (Fig. 1), by gel shift assays with avian B lymphoma cell extracts (Ruddell *et al.,* 1989). This b region (−145 to −155 bp) binds two factors (b and b*) that are expressed in many avian cell types (Ruddell *et al.,* 1989). The responsible binding motifs have not been identified, and little more is known about the contribution of these activities to LTR transcription.

An inducible LTR binding activity has been identified which is increased after Marek's disease herpesvirus infection of chick embryo fibroblasts (CEF). The PRE binding activity could be encoded by the Marek's virus or it could represent a host cell protein induced by viral infection (Banders and Coussens, 1994). The LTR region involved has been localized to a 20-bp region (−140 to −120 bp) containing PRE motifs (GGTGG) on each side of the 5′ Y box element (Fig. 1). The interaction between the proteins binding the overlapping PRE and Y box elements has not yet been examined. The inducible PRE binding activity is of interest because Marek's virus infection increases ALV expression threefold in CEF (Pulaski *et al.,* 1992) and because certain Marek's virus strains augment ALV induction of B lymphoma (Bacon *et al.,* 1989).

TRANSCRIPTION ACTIVITY OF LTR PROTEIN BINDING SITES

The sequences important for LTR-enhanced transcription have been identified by transient transfection and retroviral infection assays, using mutated LTR-reporter gene constructs expressed in avian or mammalian fibroblasts. Initially, large deletions and insertions were used to roughly define the enhancer and promoter regions (Cullen *et al.,* 1985b; Laimins *et al.,* 1984). These studies identified two regions that are important for transcription activity. Deletion of the RSV LTR enhancer region 5′ of the *Sph*I site removes the multiple CCAAT/enhancer ele-

TABLE 1

CHARACTERISTICS OF LTR BINDING PROTEINS

Factor[a]	Species[b]	Classification[c]	Binding motif[d]	References
a1	Chicken	—	TN($^{A}/_{T}$)TGCAAN	Bowers and Ruddell, 1992
A1/EBP	Chicken	bZIP	TN($^{A}/_{T}$)TGCAAN	Bowers and Ruddell, 1992
C/EBPα	Rat	bZIP	T($^{T}/_{G}$)NNG($^{C}/_{T}$)AA($^{T}/_{G}$)	Johnson *et al.*, 1987 Ryden and Beemon, 1989
EFII	Chicken	—	—	Sealy and Chalkley, 1987
E2BP	Quail	—	TGCAA($^{T}/_{G}$)A($^{C}/_{T}$)	Kenny and Guntaka, 1990
FIII	Chicken	—	—	Goodwin, 1988
Ig/EBP	Mouse	bZIP	T($^{T}/_{G}$)NNG($^{C}/_{T}$)AA($^{T}/_{G}$)	Roman *et al.*, 1990
NF/IL6	Human	bZIP	T($^{T}/_{G}$)NNGNAA($^{T}/_{G}$)	Akira *et al.*, 1990 Sears and Sealy, 1994
a3	Chicken	—	TT($^{G}/_{A}$)CATAAG	Smith *et al.*, 1994
FI	Chicken	—	—	Goodwin, 1988
VBP	Chicken	bZIP	TT($^{G}/_{A}$)CATAAG	Iyer *et al.*, 1991 Smith *et al.*, 1994
EFIII	Chicken	—	CC($^{A}/_{T}$)$_6$GG	Boulden and Sealy, 1992
E3BP	Quail	—	—	Kenny and Guntaka, 1990
RSV-d	Chicken	—	CC($^{A}/_{T}$)$_6$GG	Zachow and Conklin, 1992
b/b*	Chicken	—	—	Ruddell *et al.*, 1989
c	Chicken	—	—	Ruddell *et al.*, 1988
CBF-A/CBF-B	Rat	HAP2/HAP3	ATTGG	Maity *et al.*, 1990 Vuorio *et al.*, 1990
EFIa/EFIb	Chicken	—	ATTGG	Faber and Sealy, 1990
FII	Chicken	—	—	Goodwin, 1988
YB-1/EFI	Chicken	—	—	Grant and Deeley, 1993 Kandala and Guntaka, 1994
PRE	Chicken	—	GGTGG	Banders and Coussens, 1994

[a] LTR binding factors identified using crude nuclear extracts or purified proteins. Factors binding the same LTR region are grouped together.
[b] Species of origin.
[c] Structural classification of proteins for which cloned sequences are available.
[d] Binding motif, where characterized.

ments and one CArG box (Fig. 1), and gives a roughly 90% decrease in LTR reporter gene transcription in avian fibroblasts (Cullen *et al.*, 1985b; Norton and Coffin, 1987; Gowda *et al.*, 1988). The region 3′ of the *Sph*I site that includes the Y box and PRE motifs is also important for LTR-driven transcription. Deletions or insertions in this region decrease LTR-driven transcription by up to 90% in avian fibroblasts.

The contribution of individual LTR protein binding elements to LTR transcription activity has been analyzed. Point mutations introduced into the 5′ LTR CCAAT/enhancer elements decrease RSV LTR-driven transcription 10–50% in transient transfections or retroviral vector infection experiments in CEF (Ryden *et al.*, 1993). Combinations of mutations in two of the three LTR CCAAT/enhancer elements give a 70% decrease in LTR-driven transcription, suggesting that the multiple copies of these elements are important for LTR transcription enhancer function. Moreover, six copies of the RSV LTR CCAAT/enhancer element are able to enhance transcription of a minimal LTR promoter up to 40-fold in CEF (Sears and Sealy, 1992). The CCAAT/enhancer elements in the internal *gag* gene enhancer of the RSV provirus also contribute to the overall level of viral expression in infected fibroblasts, as mutation of this region decreases viral expression by 50% (Karnitz *et al.*, 1987; Ryden *et al.*, 1993). Mutations of the CCAAT/enhancer elements abolish binding of several bZIP factors *in vitro*, including a1/EBP (Bowers and Ruddell, 1992), C/EBPα (Ryden *et al.*, 1993), and VBP (Smith *et al.*, 1994), supporting the idea that these factors are important for LTR enhancer activity.

Point mutations in the LTR CArG boxes decrease transcription of synthetic LTR constructs roughly 50% in rat fibroblasts (Lang *et al.*, 1993), indicating that the CArG boxes and associated binding proteins are important for basal LTR function. These elements may mediate the fivefold increase in RSV LTR-driven transcription observed after serum treatment of avian or mammalian fibroblasts (Boulden and Sealy, 1990; Dutta *et al.*, 1990). The isolated CArG box element confers serum responsiveness to a minimal LTR promoter construct, giving a threefold increase in transcription activity after serum treatment of CEF (Boulden and Sealy, 1992).

The LTR Y box elements appear to be essential for LTR promoter function, as mutations in the 5′ or 3′ Y box elements abolish protein binding activity and decrease

LTR-driven transcription up to 99% in transient transfection assays (Greuel *et al.,* 1990). The Y box binding factors purified from murine fibroblasts activate the RSV LTR promoter in an *in vitro* transcription system, supporting the idea that protein binding to these elements is involved in LTR promoter function (Maity *et al.,* 1988). The Y box elements could also contribute to the serum responsiveness of LTR-driven transcription in certain transformed mammalian fibroblast cell lines (Dutta *et al.,* 1990), although in other fibroblast lines serum responsiveness is regulated primarily by the CArG box elements (Lang *et al.,* 1993).

Marek's disease virus infection increases LTR-driven transcription in ALV-infected CEF and also increases the activity of the PRE LTR binding factor *in vitro* (Pulaski *et al.,* 1992; Banders and Coussens, 1994). Deletion of the 20-bp LTR region including the PRE and Y box elements (−140 to −120 bp) decreases basal LTR transcription, and also reduces the Marek's disease virus enhancement of transcription. The relative contributions of the PRE and Y box elements to these effects have not been determined. However, the correlation of PRE binding activity with increased LTR transcription suggests that this factor may be involved in the interaction of the Marek's virus with ALV.

CONCLUSIONS

A number of complementary studies have shown that several LTR protein binding sites contribute to regulating high levels of LTR transcription in avian and mammalian cells. The Y box elements appear to be essential for LTR promoter function, while the CCAAT/enhancer elements are important for enhancer function. The CArG box and PRE elements may mediate modulation of LTR activity by growth factors or by viral transactivators, respectively. These elements are all present in more than one copy in the ALV and RSV LTRs, and this repetitive organization may be important to drive high rates of LTR-enhanced transcription. The transcriptionally inactive endogenous retrovirus LTRs lack the CCAAT/enhancer elements and contain only one CArG box and Y box relative to the exogenous ALV and RSV virus LTRs, due to extensive deletions in the endogenous virus LTR (Zachow and Conklin, 1992). Interestingly, if a second CArG box or Y box motif from the RSV LTR is introduced into the endogenous virus LTR it gains significant transcription activity (Habel *et al.,* 1993). This suggests that pairs of elements are required for maximal LTR function. Repetitive protein binding motifs are a common feature in retroviral LTRs, and this repetitive structure contributes to viral oncogenic potential. For example, duplication of the LTR enhancer in the mink cell focus forming virus is important for leukemogenicity (Holland *et al.,* 1989). The human immunodeficiency virus (HIV) type 1 LTR contains a duplicated NFkB element relative to HIV-2, which may mediate the differential transcription response of these viruses to T cell activation signals (Hannibal *et al.,* 1993).

The ALV and RSV U3 LTR regions are very similar, showing about 80% sequence identity (Bizub *et al.,* 1984). Most of the sequence variation occurs in regions between the protein binding sites (Fig. 1), supporting the idea that protein binding to these conserved sites is important for viral transcription. The multiple proteins interacting with the ALV and RSV strains are the same for the most part. The major binding site difference observed is a 5-bp deletion in the SR-RSV-A strain, which abolishes binding of the VBP bZIP factor to this site (Smith *et al.,* 1994). The 5′ CArG box element also shows a point mutation from T to C in the AT rich core region of the RSV strains (Fig. 1) which could influence the serum-responsive activity of this element (Lang *et al.,* 1993). These LTRs all drive high levels of transcription, suggesting that the protein binding differences have little effect. However, the transcription activity of the LTRs has not been directly compared, leaving open the possibility that they show subtle differences in transcription activity or oncogenic potential.

The RSV LTR is expressed well in birds and in mammals and for the most part appears to use the same factor binding sites in each species. One interesting difference is that while most proviral LTRs are active in avian cells, only a very small number of integrated proviruses are expressed in infected rat embryo fibroblasts (Wyke and Poole, 1990). This does not appear to be due to limited availability of LTR binding factors in rat cells (Lang *et al.,* 1993). Instead, the transcription silencing appears to be regulated in some way by the site and structure of the proviral integration site (Fincham and Wyke, 1991, 1992). The transcription potential of the endogenous avian viruses also appears to be influenced by chromatin structure and DNA methylation at the site of proviral integration (Conklin *et al.,* 1982; Conklin and Groudine, 1986).

The avian retroviruses are transcribed at high levels in many tissues (Ruddell *et al.,* 1988; Robinson *et al.,* 1993). This probably reflects the contributions of binding factors from several transcription factor families (Table 1). Of the CCAAT/enhancer element binding proteins, C/EBPα is restricted to liver and other differentiated cell types (Birkenmeier *et al.,* 1989), while NF/IL6 is restricted to certain activated cell types (Akira *et al.,* 1990). The Ig/EBP factor is more widely expressed (Roman *et al.,* 1990), as is VBP (Iyer *et al.,* 1991), and these factors could be used to regulate LTR transcription enhancement in many cell types. The CArG box factors have primarily been studied in fibroblasts, and it remains to be determined if these factors influence transcription in other cell types. The Y box binding proteins are expressed in many cell types (Faber and Sealy, 1990; Grant and Deeley, 1993). All of these factors may contribute (perhaps in different combinations) to the widespread high activity of this transcription unit in different species. The mechanism by which these proteins interact with RNA polymerase II to direct high level transcription remains to be determined.

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