Differential DNA Binding of Nuclear Proteins to a Long Terminal Repeat Region of the MCF13 and Akv Murine Leukemia Viruses

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Long terminal repeat (LTR) sequences of murine leukemia viruses (MLVs) have been demonstrated to be mainly responsible for the pathogenic differences in these retroviruses. A region of the LTR which is downstream of the enhancer elements has been shown to contribute both to enhancer activity as well as to disease specificity of MLVs. We have identified protein-DNA complexes generated by this region of a lymphomagenic MLV (MCF13) and one which is nonpathogenic (Akv). One protein-DNA complex we have observed for this region is unique to MCF13 DNA sequences. Detection of protein involved in this unique MCF13 complex in different cell lines revealed that it was ubiquitous.

The primary genetic determinant of the tissue-specific type of neoplasia produced by different murine replication-competent retroviruses is the long terminal repeat (LTR) region of the viral genome as shown by studies involving recombinant retroviruses and transient expression assays on cells in culture (2-4, 6-8, 13, 15, 17, 20, 21, 23, 25). Molecular dissections of viral LTRs have identified the regions that contribute to tissue-specific transcriptional activity and pathogenicity of recombinant viruses. These regions include the tandem direct repeats that function as enhancer elements and sequences downstream of these repeats (2, 11, 12, 16, 18). We have recently shown that the latter sequences and 18 base pairs (bp) of the enhancer of the LTR of a lymphomagenic murine retrovirus (MCF13) produced nearly as great cell-type-specific transcriptional activity as an intact LTR (14). Because of this observation, we wished to determine whether *trans*-acting factors important for transcriptional activity could bind to the sequences downstream of the enhancer of the MCF13 LTR and whether there is differential binding compared with this same region of the LTR of the nonpathogenic Akv virus. Identification of these cellular factors may help to explain the tissue-specific pathogenicity of the MCF13 virus.

The region of the MCF13 and Akv LTRs which we have used to detect the potential binding of nuclear proteins by the gel-shift assay (10) is located primarily immediately downstream of the tandem repeat sequences of these two viruses and upstream of both distal and proximal promoter elements (CAAT and TATA boxes, respectively) (Fig. 1A). For the MCF13 LTR, we have used a 99-bp *RsaI*-*Bam*HI DNA fragment (dMCF) containing nucleotide sequences between the *RsaI* and *DraI* sites in addition to 6 bp of polylinker from pUC18. A 96-bp *AvaII*-*DraI* DNA fragment (dAkv) corresponding to a similar region of the Akv LTR was also used in this study (Fig. 1B).

When nuclear extract (9) from a murine T-lymphoma cell line (L691) was used to bind ³²P-labeled dMCF, we observed a complex electrophoretic pattern consisting of five different bands (Fig. 2A, I through V) in a gel-shift assay (10). The addition of increasing amounts of protein to the binding reaction yielded proportionately greater amounts of protein-DNA complexes (Fig. 2A, lanes 2 to 6). This cell line was used for the preparation of nuclear extract because we had

observed for it that the MCF LTR was more active than the Akv in a transient expression assay (14). To determine whether these protein-DNA complexes corresponded to specific binding to this fragment, we performed DNA competition experiments with either unlabeled dMCF or a 200-bp pUC18 DNA fragment representing nonspecific DNA (Fig. 2B). Increasing amounts of unlabeled dMCF DNA were added to 1 ng of ³²P-labeled dMCF in a binding reaction experiment (lanes 2 to 5). At a molar ratio of 100:1 of unlabeled versus labeled DNA, competition was effectively complete for complexes II through V with the dMCF fragment (Fig. 2B, lane 5). Although we did not detect complete competition for complex I at the highest molar ratio included in this figure, we were able to eliminate this band entirely with larger amounts of competing unlabeled dMCF (data not shown). Nonspecific pUC18 DNA at similar molar ratios competed only for complexes IV and V (Fig. 2B, lanes 6 to 9), indicating that these complexes have arisen from nonspecific binding of protein to this DNA fragment.

Binding reactions with the dAkv fragment produced protein-DNA complexes with similar mobilities to four of the five complexes detected for dMCF (Fig. 3A). We did not observe a dAkv protein-DNA complex that comigrated with dMCF complex II. A longer exposure of these autoradiograms (lanes 3 and 4) demonstrated that our inability to detect complex II for dAkv was not due to its presence at a low level. We also observed that complex formation with dAkv DNA was dependent on protein concentration (Fig. 3B).

Although the dAkv and dMCF protein-DNA complexes had similar mobilities, competition experiments revealed that they probably involved the binding of different proteins. Competition experiments with increasing amounts of unlabeled DNA fragments revealed that although dAkv could effectively compete with all four complexes (Fig. 3C, lanes 3 to 5), only complex V could be competed for by pUC18 DNA (Fig. 3C, lanes 6 to 8). Similar experiments involving dMCF as the competitor DNA also demonstrated significant competition only for dAkv complex V (lanes 9 to 11). These results suggest that either different proteins may be involved in the generation of the specific protein-DNA complexes I, III, and IV detected for these two viral DNA fragments or the proteins are similar but have different binding affinities for these DNAs.

We wished to localize the protein-binding site for the

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A.

MCF 13 LTR

B.

FIG. 1. MLV LTR. (A) A 600-bp MCF13 LTR; open boxes are 69-bp direct repeats; hatched box delineates the downstream *RsaI*-*DraI* DNA fragment used in this study; CAT and TATA boxes are indicated; rightward arrow marks transcription initiation site. (B) Comparison of nucleotide sequences downstream of the enhancer element for MCF13 and Akv MLVs; nucleotide differences are shown; blank spaces correspond to identical bases; the dot refers to a deletion. The 3' end of each viral enhancer is indicated by a bracket. Restriction endonuclease sites used to generate DNA fragments for this study are indicated.

unique protein-DNA complex II that we have observed for dMCF but not for dAkv. We have hypothesized that because of the uniqueness of this complex to dMCF DNA, it may be important for the difference in cell-type-specific transcription we have detected for the MCF13 and Akv LTRs (25). To start to localize the site of protein binding, we used subfragments of the dMCF DNA in a competition experiment using the gel-shift assay. For competition we used DNA fragments corresponding to the 5' or 3' half of the dMCF fragment that resulted from cleavage with *DdeI* (Fig. 1B). A 45-bp fragment consisting of the 5' half of dMCF effectively eliminated complex II at a molar ratio of cold competitor-to-labeled DNA of 10:1 (Fig. 4, lane 3). As shown in lane 5, the same amount of a 54-bp DNA fragment corresponding to the 3' half of dMCF showed no competition with complex II. These data suggested that the site of protein binding involved in the generation of dMCF complex II resided upstream of the *DdeI* site.

Because of the possibility that the protein(s) involved in dMCF complex II may be cell-type specific, we screened various cell lines for the presence of protein which could generate this complex. Results of gel-shift assays of the dMCF fragment reacted with nuclear extracts from different cell types are presented in Fig. 5. The different murine cell lines used to prepare nuclear extracts included T-cell lines (L691, SL3, and S49), B-cell lines (P3 and WEHI 231), and an NIH 3T3 fibroblast line. We also examined a human carcinoma cell line (MCF-7). We observed that protein-DNA complexes with similar mobilities to that of dMCF complex II were generated by nuclear extracts from all of the cell lines examined. We also observed protein-DNA complexes with similar mobilities to those of the two other dMCF-specific complexes (I and III) with these nuclear extracts. There were differences for some of the lines compared with L691, however, which included different relative band intensities (e.g., P3, lane 4) or the presence of additional bands (e.g., WEHI 231, lane 5). Our analysis of these different cell types suggested that the nuclear proteins generating dMCF

FIG. 2. (A) Titration of protein-DNA complexes generated by the gel-retardation assay for the dMCF DNA fragment with L691 nuclear extract. Five different protein-DNA complexes are indicated by Roman numerals. Lanes (micrograms of protein): 1, 0; 2, 0.5; 3, 1.0; 4, 2.0; 5, 4.0; 6, 6.0. dMCF (99 bp) was ^{32}P -labeled with T4 DNA polymerase and the large fragment of *Escherichia coli* DNA polymerase I, using $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ and $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$, and was purified by polyacrylamide gel electrophoresis. One nanogram of the labeled fragment was incubated with various amounts of L691 nuclear extract at room temperature for 30 min with 1 μg of poly(dI-dC) from Boehringer Mannheim in 12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-NaOH (pH 7.9)-25 mM NaCl-0.5 mM EDTA-1 mM dithiothreitol. Protein-DNA complexes were separated by electrophoresis through a 4% polyacrylamide gel with a running buffer of 6.7 mM Tris hydrochloride (pH 7.5)-1 mM EDTA-3.3 mM sodium acetate for 90 min at room temperature. After electrophoresis, gels were dried and exposed to X-ray film at -70°C with an enhancing screen. Nuclear extract from L691 cells was prepared as described by Fujimura (9). (B) Binding competition for ^{32}P -labeled dMCF. Ratios of unlabeled to labeled DNA were 0:1, 1:1, 10:1, 50:1, and 100:1 for unlabeled dMCF (lanes 1 to 5); and 1:1, 10:1, 50:1, and 100:1 for unlabeled pUC DNA (lanes 6 to 9). Competition experiments were performed by adding both labeled and unlabeled DNA at the same time to the reaction mixture containing 4 μg of L691 nuclear extract-1 μg poly(dI-dC)-buffer before incubation at room temperature for 30 min.

FIG. 3. (A) Comparison of the protein-DNA complexes generated by the dMCF and dAkV DNA fragments. dAkV corresponds to a 96-bp *AvaII-DraI* DNA fragment. DNA labeling, protein binding, and polyacrylamide gel electrophoresis conditions were identical to those described for dMCF in the legend to Fig. 2A. Four micrograms of protein was used in these binding reactions. Gel-retardation electrophoretic patterns for dAkV (lane 1) and dMCF (lane 2) are shown. Lanes 3 and 4 represent a longer exposure of lanes 1 and 2. Roman numerals correspond to the protein-DNA complexes observed for dMCF. (B) Protein titration of the dAkV DNA fragment. Various amounts of L691 nuclear extract were reacted with 1 ng of 32 P-labeled dAkV DNA and electrophoresed through a 4% polyacrylamide gel as detailed in the legend to Fig. 2. Lanes (micrograms of protein): 1, 0; 2, 0.5; 3, 1.0; 4, 2.0; 5, 4.0; 6, 6.0. (C) Binding competition for 32 P-labeled dAkV with unlabeled DNAs. Conditions for competition reactions and gel electrophoresis are as described in the legend to Fig. 2. Lane 1 contains labeled dAkV with no protein. Shown are unlabeled to labeled DNA ratios of 0:1, 1:1, 10:1, and 50:1 for unlabeled dAkV (lanes 2 to 5); 1:1, 10:1, and 50:1 for unlabeled pUC DNA (lanes 6 to 8); and 1:1, 10:1, and 50:1 for unlabeled dMCF (lanes 9 to 11).

complexes I, II, and III were ubiquitous and were present in different species.

We have previously demonstrated that sequences in the LTR of a mink cell focus-forming murine leukemia virus (MCF13 MLV), which induces thymic lymphomas, can potentiate gene expression to a greater degree in T lymphocytes compared with that in fibroblasts (25). This was in contrast to the LTR from a nonpathogenic MLV (AkV) which had equal activities in these cell types. We have suggested that this cell-type-specific activity of the MCF LTR may contribute to the type of neoplasia involved with

this class of murine retroviruses. In this report, we have further analyzed the role of the sequences downstream of the enhancer of MCF13 MLV because studies have shown that these sequences contribute to disease specificity (11, 15, 18) and to the transcriptional activity of the MCF13 LTR in T cells (14).

It is now a well-accepted idea that *trans*-acting factors that bind to specific DNA sequences involved in transcriptional regulation are essential for the regulatory function of these *cis*-acting sequences. We have, therefore, searched for *trans*-acting factors which may interact with the region 3' to the MCF13 (dMCF) and AkV (dAkV) enhancers in a differential manner, thereby accounting for the different cell-type activities we have observed for these two LTRs. By gel-shift assays, we have observed three specific protein-DNA complexes generated by an MCF13 DNA fragment correspond-

FIG. 4. Localization of dMCF protein-DNA complex II to the 5' half of the dMCF fragment by binding competition. DNA fragments of 45- and 54-bp, corresponding to the 5' and 3' half, respectively, of the dMCF DNA were generated by *DdeI* digestion of dMCF. These unlabeled DNAs were used to compete with 32 P-labeled dMCF in a binding reaction as described in the legend to Fig. 2B. Lane 1 represents 32 P-dMCF with no protein in the binding reaction. Lanes: 2 and 3, 0:1 and 10:1 ratios, respectively, of the unlabeled 5' half of dMCF to labeled dMCF; 4 and 5, 0:1 and 10:1 ratios, respectively, of the unlabeled 3' half of dMCF DNA to labeled dMCF.

FIG. 5. Binding of nuclear extracts from different cell lines to 32 P-labeled dMCF. Murine T-cell lines L691 (lane 1), SL3 (lane 2), and S49 (lane 3); murine B-cell lines P3 (lane 4) and WEHI 231 (lane 5); murine fibroblast cell line, NIH 3T3 (lane 6); human carcinoma cell line, MCF-7 (lane 7). Gel-retardation assays were performed as detailed in the legend to Fig. 2. Four micrograms of protein was used in the binding reaction for all the cell lines except for P3 (6 μ g) and NIH 3T3 (6 μ g).

ing to this region of the LTR. Of these, complex II was detected only for dMCF.

Although we have observed differential binding of nuclear factors to the MCF13 and Akv LTR regions 3' to their enhancers, we have determined that these factors are present in all the different cell types we have examined and do not represent cell-specific proteins that have been detected for some cellular genes (19, 24). How then do these sequences in the MCF13 LTR potentiate transcription to a greater extent in T cells? One possibility is that different combinatorial arrangements of ubiquitous proteins bound to upstream regulatory sequences interact with different effectiveness with similar or different promoter complexes depending on the cell type. There is evidence that an enhancer can increase transcription from different promoters to different degrees (1). These combinatorial arrangements would be dictated by different DNA sequences present in transcriptional regulatory regions. Our observations that there are ubiquitous *trans*-acting factors which bind differentially to the MCF13 and Akv LTRs would support this hypothesis. A second possibility is that protein-protein interactions which are undetectable by the gel-retardation assay we have used in this study will be responsible for cell-type-specific transcriptional activity. Other possible explanations include the binding to the same DNA sequence of similar but different *trans*-acting factors encoded by a multigene family and present in different cell types, for which there is precedence (5, 22), or different posttranslational modifications in different cell types of the same cellular protein that are not detectable by the gel-shift assay. Either the purification of this DNA-binding protein(s) or the isolation of the gene(s) which encodes it will be required for a definitive answer.

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LITERATURE CITED

- Berg, P. E., Z. Popovic, and W. F. Anderson. 1984. Promoter dependence of enhancer activity. *Mol. Cell. Biol.* 4:1664-1668.
- Bosze, Z., H.-J. Thiesen, and P. Charnay. 1986. A transcriptional enhancer with specificity for erythroid cells is located in the long terminal repeat of the Friend murine leukemia virus. *EMBO J.* 5:1615-1623.
- Celander, D., and W. A. Haseltine. 1984. Tissue-specific transcription preference as a determinant of cell tropism and leukemogenic potential of murine retroviruses. *Nature (London)* 312:159-162.
- Chatis, P. A., C. A. Holland, J. W. Hartley, W. P. Rowe, and N. Hopkins. 1983. Role for the 3' end of the genome in determining disease specificity of Friend and Moloney murine leukemia viruses. *Proc. Natl. Acad. Sci. USA* 80:4408-4411.
- Curran, T., and B. R. Franza, Jr. 1988. *fos* and *jun*: the AP-1 connection. *Cell* 55:395-397.
- DesGroseillers, L., and P. Jolicoeur. 1984. The tandem direct repeats within the long terminal repeat of murine leukemia viruses are the primary determinant of their leukemogenic potential. *J. Virol.* 52:945-952.
- DesGroseillers, L., and P. Jolicoeur. 1984. Mapping the viral sequences conferring leukemogenicity and disease specificity in Moloney and amphotropic murine leukemia viruses. *J. Virol.* 52:448-456.
- DesGroseillers, L., E. Rassart, and P. Jolicoeur. 1983. Thymotropism of murine leukemia virus is conferred by its long terminal repeat. *Proc. Natl. Acad. Sci. USA* 80:4203-4207.
- Fujimura, F. K. 1986. Nuclear activity from F9 embryonal carcinoma cells binding specifically to the enhancers of wild-type polyoma virus and PyEC mutant DNAs. *Nucleic Acids Res.* 14:2845-2861.
- Garner, M. M., and A. Revzin. 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the *Escherichia coli* lactose operon regulatory system. *Nucleic Acids Res.* 9:3047-3060.
- Golemis, E., Y. Li, T. N. Frederickson, J. W. Hartley, and N. Hopkins. 1989. Distinct segments within the enhancer region collaborate to specify the type of leukemia induced by nondefective Friend and Moloney viruses. *J. Virol.* 63:328-337.
- Holland, C. A., P. Anklesaria, M. A. Sakakeeny, and J. S. Greenberger. 1987. Enhancer sequences of a retroviral vector determine expression of a gene in multipotent hematopoietic progenitors and committed erythroid cells. *Proc. Natl. Acad. Sci. USA* 84:8662-8666.
- Holland, C. A., J. Wozney, P. A. Chatis, N. Hopkins, and J. W. Hartley. 1985. Construction of recombinants between molecular clones of murine retrovirus MCF 247 and AKV: determinant of an in vitro host range property that maps in the long terminal repeat. *J. Virol.* 53:152-157.
- Hollon, T., and F. K. Yoshimura. 1989. Mapping of functional regions of murine retrovirus long terminal repeat enhancers: enhancer domains interact and are not independent in their contributions to enhancer activity. *J. Virol.* 63:3353-3361.
- Ishimoto, A., M. Takimoto, A. Adachi, M. Kakuyama, S. Kato, K. Kakimi, K. Fukuoka, T. Ogiu, and M. Matsuyama. 1987. Sequences responsible for erythroid and lymphoid leukemia in the long terminal repeats of Friend-mink cell focus-forming and Moloney murine leukemia viruses. *J. Virol.* 61:1861-1866.
- Laimins, L. A., P. Gruss, R. Pozzatti, and G. Khoury. 1984. Characterization of enhancer elements in the long terminal repeat of Moloney murine sarcoma virus. *J. Virol.* 49:183-189.
- Lenz, J., D. Celander, R. L. Crowther, R. Patarca, D. W. Perkins, and W. A. Haseltine. 1984. Determination of the leukemogenicity of a murine retrovirus by sequences within the long terminal repeat. *Nature (London)* 308:467-469.
- Li, Y., E. Golemis, J. W. Hartley, and N. Hopkins. 1987. Disease specificity of nondefective Friend and Moloney murine leukemia viruses is controlled by a small number of nucleotides. *J. Virol.* 61:693-700.
- Nelson, C., V. R. Albert, H. P. Elsholtz, L. I.-W. Lu, and M. G. Rosenfeld. 1988. Activation of cell-specific expression of cat growth hormone and prolactin genes by a common transcription factor. *Science* 239:1400-1405.
- Oliff, A., K. Signorelli, and L. Collins. 1984. The envelope gene and long terminal repeat sequences contribute to the pathogenic phenotype of helper-independent Friend viruses. *J. Virol.* 51:788-794.
- Rosen, C. A., W. A. Haseltine, J. Lenz, R. Ruprecht, and M. W. Cloyd. 1985. Tissue selectivity of murine leukemia virus infection is determined by long terminal repeat sequences. *J. Virol.* 55:862-866.
- Santoro, C., N. Mermod, P. C. Andrews, and R. Tjian. 1988. A family of human CCAAT-box-binding proteins active in transcription and DNA replication: cloning and expression of multiple cDNAs. *Nature (London)* 334:218-224.
- Short, M. K., S. A. Okenquist, and J. Lenz. 1987. Correlation of leukemogenic potential of murine retroviruses with transcriptional tissue preference of the viral long terminal repeats. *J. Virol.* 61:1067-1072.
- Staudt, L. M., H. Singh, R. Sen, T. Wirth, P. A. Shays, and D. Baltimore. 1986. A lymphoid-specific protein binding to the octamer motif of immunoglobulin genes. *Nature (London)* 323:640-643.
- Yoshimura, F. K., B. Davison, and K. Chaffin. 1985. Murine leukemia virus long terminal repeat sequences can enhance gene activity in a cell-type-specific manner. *Mol. Cell. Biol.* 5:2832-2835.