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# Regulatory Elements within the Murine Leukemia Virus Enhancer Regions Mediate Glucocorticoid Responsiveness

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**Enhancer elements within nonleukemogenic (Akv) and T-cell leukemogenic (SL3-3) murine leukemia viruses demonstrate strong cell type preference in transcriptional activity. These transcription elements are additionally regulated by the synthetic glucocorticoid dexamethasone, and this pattern of regulation varies according to cell type. The sequences required for dexamethasone regulation for both Akv and SL3-3 are shown to include a 17-nucleotide consensus sequence previously termed the glucocorticoid response element (GRE). Although the GREs are identical for both viral enhancers, the sequences surrounding these elements differ, as does the spatial arrangement of the GRE sequences with respect to one another. It is proposed that the spatial arrangement of the GREs, as well as their precise sequence context, determines the difference in the response to dexamethasone of the enhancers in different cell types.**

Murine leukemia viruses compose a family of retroviruses that induce a variety of cell-specific diseases in mice. One major viral determinant of disease specificity is located within the U3 region of the long terminal repeat (LTR) of the viral genome (6, 7, 10, 11, 16, 21, 22). The disease specificity of an isogenic set of viruses that differ in only their LTR sequences is correlated with the ability of these viruses to replicate in specific cell types (35). Analysis of transcription element function of the U3 regions from the LTRs of these isolates in different cell types in vitro (1, 3, 4, 23, 40, 49) suggests that the differential activity of the viral transcriptional enhancer is responsible for the observed cell tropism and leukemogenicity of these viruses in vivo.

In an earlier report (4), it was noted that the tandemly duplicated regions of the Akv and SL3-3 viral LTRs contain multiple 17-nucleotide sequences that are identical to the consensus sequence (5, 18, 31, 38) identified as the glucocorticoid response element (GRE) (5) (Fig. 1). Moreover, it was shown that expression of the gene encoding the chloramphenicol acetyltransferase protein (CAT) directed by the viral LTRs was stimulated by dexamethasone in both HeLa cells and T cells (4). In HeLa cells, the constitutive level of CAT enzyme activity directed by the Akv LTR was higher than that directed by the SL3-3 LTR. However, in the presence of dexamethasone the level of CAT enzyme activity directed by both LTRs was similar. The activity of the Akv and SL3-3 LTRs was increased by the hormone approximately 5- and 25-fold, respectively (4). In the work described in this report, we examined whether the GRE consensus sequences are required for the response to dexamethasone and explored the basis for the differences in activity of the two LTR sequences.

## MATERIALS AND METHODS

**Recombinant plasmids.** The plasmids pRSVCAT (13), pAU3CAT (3), pSU3CAT (3), and pSV1XCAT (3) have been described previously.

The strategy used to construct all the plasmids relied on the use of *Sal*I and *Xho*I synthetic linker restriction enzyme sites at the endpoints and junctions of all regulatory sequences in the native and hybrid LTRs. All of the Akv and SL3-3 U3 region 5′ deletion mutants were generated by using the natural restriction enzyme sites present in each LTR (Fig. 2a). For example, the mutant plasmids with endpoints at nucleotide positions −231, −208, and −184 (relative to the viral start site as +1) were generated by using *Eco*RV, *Apa*I, and *Ava*II, respectively. Two of these plasmids [Fig. 2b, p(5′-208)AU3CAT and p(5′-184)AU3CAT] were generated by using an enzyme that cleaves elsewhere in the vector. In these cases, the DNAs were digested with *Xho*I and *Bam*HI following addition of the synthetic *Xho*I linker, and the fragment containing the CAT gene and the LTR sequences was gel purified and ligated to the *Xho*I-*Bam*HI fragment which contained the vector portion of the original parent plasmid (Fig. 2a, pAU3CAT).

A single copy of 99-nucleotide monomer and a copy of a 72-nucleotide monomer from the Akv and SL3-3 repeat regions, respectively, were deleted by using *Eco*RV, which cleaves each repeat region twice (Fig. 2a, −330 and −231 for Akv and −303 and −231 for SL3-3). These digested DNAs were gel isolated and recircularized in the presence of T4 DNA ligase to produce p(3′-330/5′-231)AU3CAT and p(3′-303/5′-231)SU3CAT, respectively (Fig. 2b and c). An 8-nucleotide synthetic *Sal*I linker was introduced at nucleotide positions −330 and −231 for Akv and −303 and −231 for SL3-3 prior to recircularization of *Eco*RV-digested pAU3 CAT and pSU3CAT in the presence of T4 DNA ligase. These plasmids are designated p(3′-330/S/5′-231)AU3CAT and p(3′-303/S/5′-231)SU3CAT to indicate the insertion of the synthetic linker (Fig. 2e).

Internal deletions of pAU3CAT and pSU3CAT were constructed by using the *Sal*I-*Bam*HI fragment of p(3′-330/S/5′-231)AU3CAT and p(3′-303/S/5′-231)SU3CAT as the source of 5′ enhancer sequences and the *Xho*I-*Bam*HI fragments of the various 5′ deletion mutants as the source of the 3′ enhancer sequences, promoter sequences, and the CAT gene (illustrated in Fig. 2b and c). Finer deletions in the SL3-3 repeat region were made in the following manner. The SL3-3 LTR was subcloned as a *Xho*I-*Hin*dIII fragment in a derivative of pBR322 that contains a unique *Xho*I site at nucleotide position 4361 (denoted as pBRX322), and the resultant plasmid was denoted as pBRXSU3. This plasmid

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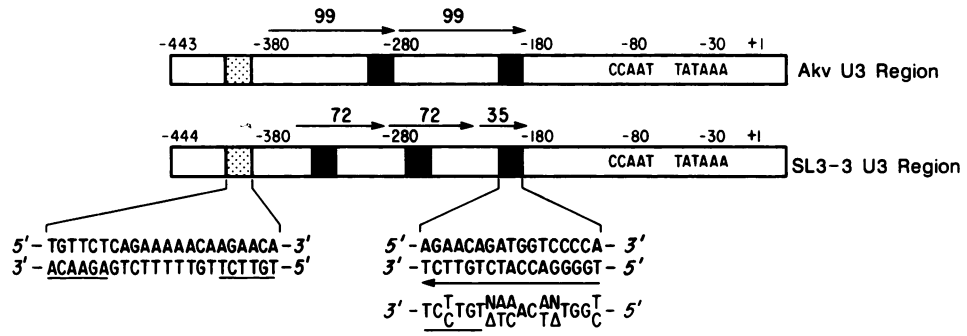



FIG. 1. Organization of consensus GRE sequences within the murine enhancer regions. Symbols: ▩, location of the dyad symmetry that contains sequences homologous to the hexanucleotide core consensus element; ■, location of sequences that share more extensive homology with regulatory sequences present in known glucocorticoid-regulated transcriptional elements. The nucleotide positions above the line drawing of the LTR sequences refer to the locations of these elements relative to the viral start site (denoted by +1). The sequences in these regions are identical between Akv and SL3-3 and are illustrated in comparison with the previously published consensus sequences. The hexanucleotide consensus element is present in both the stippled and solid areas and is indicated by underlined sequences.

was digested with *Apa*I (which cleaves at nucleotide positions −280 and −208 in the SL3-3 U3 region), and the linearized plasmid was digested with BAL 31 exonuclease. Aliquots of this reaction mixture were extracted with phenol-chloroform at various times after addition of the BAL 31 exonuclease. Following ethanol precipitation of the digested plasmids, the DNAs were suspended in T4 polymerase buffer and incubated with T4 DNA polymerase to flush the BAL 31 end products. These ends were converted to *Sal*I sites by incubation of the flushed DNA with 8-nucleotide synthetic *Sal*I linkers in the presence of T4 DNA ligase. The linkered DNA was digested with *Sal*I, and gel-purified vector DNAs were recircularized in the presence of T4 DNA ligase. The plasmid clones which contained a *Sal*I linker were selected for further analysis by restriction enzyme mapping, and the precise deletion endpoints were determined by the sequencing techniques of Maxam and Gilbert (27). An undefined *cis*-acting sequence present in the pBRX322 vector (between nucleotide positions 651 and 2246) inhibited transient expression of CAT genes linked to either Akv or SL3-3 U3 regions in transfected murine T cells. Although this effect was not further investigated, the phenomenon closely resembles the observations reported recently by Peterson and co-workers (32, 42). Because of the complicating effects of the pBRX322 vector in transient-expression assays, a select number of internal deletions of p(3'-280/5'-208)SU3CAT were constructed ultimately by combining the *Xho*I-*Sal*I deletion fragments generated in pBRXSU3 with the LTR sequences present in plasmid p(5'-208)AU3CAT (Fig. 2c).

Specific linker insertions within the SL3-3 LTR of pSU3CAT were made by partial digestion of pSU3CAT with either *Apa*I or *Ava*II. The ends of linearized cleavage products were repaired with T4 DNA polymerase and ligated to an 8-nucleotide synthetic *Bgl*II site linker. The location and number of inserted linkers were confirmed by restriction enzyme analysis. Plasmids that contained single linker inserts were selected for genetic analysis.

The Rous sarcoma virus (RSV)-murine hybrid LTR plasmids were constructed in the following fashion. The RSV LTR in pRSVCAT contains a unique *Sph*I site at nucleotide position −138. This site was converted to an *Xho*I site by digesting the plasmid with *Sph*I, treating the *Sph*I 3' overhang ends with T4 DNA polymerase in the presence of all four deoxynucleoside triphosphates, and incubating the flushed ends with an 8-nucleotide synthetic *Xho*I linker in the presence of T4 DNA ligase. The plasmid was digested with *Xho*I, gel purified, and recircularized in the presence of T4 DNA ligase to generate p(3'-142/X/5'-138)RSVCAT. This plasmid contains an *Xho*I site that separates the viral enhancer from its natural promoter. The various plasmids which contain the RSV enhancer ligated adjacent to different promoter deletion mutants, as well as the plasmids that contain murine enhancer sequences adjacent to the RSV promoter, were constructed by incubating the appropriate *Xho*I-*Bam*HI and/or *Sal*I-*Bam*HI fragments together in the presence of T4 DNA ligase (Fig. 2d). The plasmid p(5'-138)RSVCAT is an enhancer-deficient version of pRSVCAT (Fig. 2d) and was generated by digesting p(3'-142)RSVCAT and pSV1XCAT with *Xho*I and *Bam*HI and incubating the *Xho*I-*Bam*HI fragments containing the RSV promoter and the CAT gene with the vector portion of pSV1XCAT in the presence of T4 DNA ligase.

All plasmids were manipulated by standard recombinant DNA practices (26). All plasmids used for transient expression in mammalian cells were purified through two-cycle banding by CsCl-ethidium bromide gradient ultracentrifugation.

**Cell culture and DNA transfection.** HeLa cells were maintained in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Hazleton Research Products, Denver, Pa.). The cells were seeded at a density of $1.4 \times 10^6$ cells per 100-mm dish the day before transfection. Cell transfection was done by the DEAE-dextran procedure with slight modifications as described previously (3, 4, 24). Briefly, the cells were rinsed twice with serum-free medium and incubated for 4 h with transfection cocktail containing 1 μg of plasmid DNA per ml suspended in serum-free medium containing 0.25 mg of DEAE-dextran per ml and 50 mM Tris hydrochloride (pH 7.3). After aspiration of the transfection cocktail, the cells were incubated for 8 min with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline containing 10% dimethyl sulfoxide. After aspiration of the HEPES-buffered saline–dimethyl sulfoxide solution, the cells were rinsed with phosphate-buffered saline and refed with fresh serum containing 10% fetal bovine serum. At 24 h after addition of the DNA, one-half of the parallel-transfected cell plates were treated with 1μM (final concentration) dexamethasone (44 μl of a 100-μg/ml dexamethasone solution was added to cell plates containing 11 ml of medium). After an additional 24 h of continued transient expression,

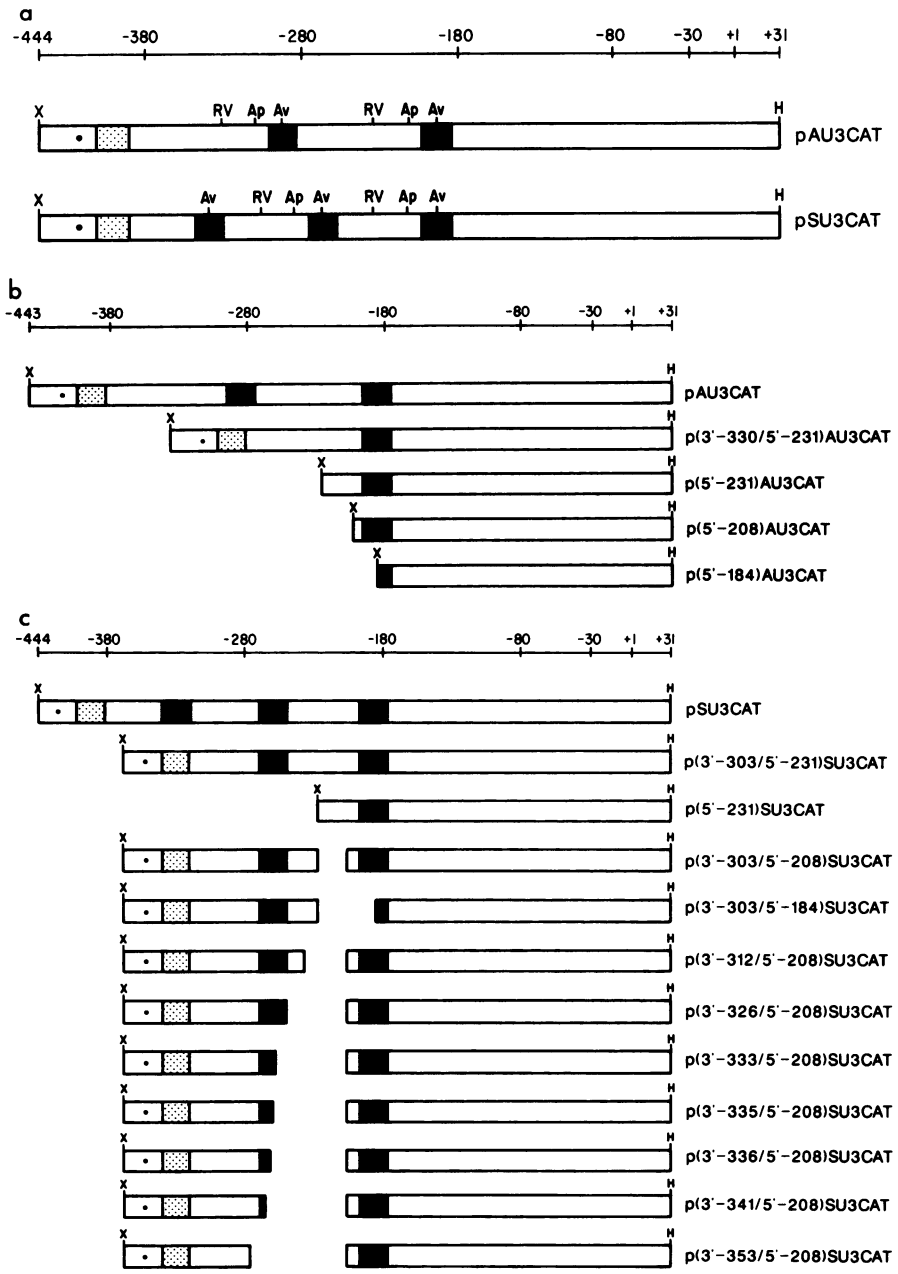
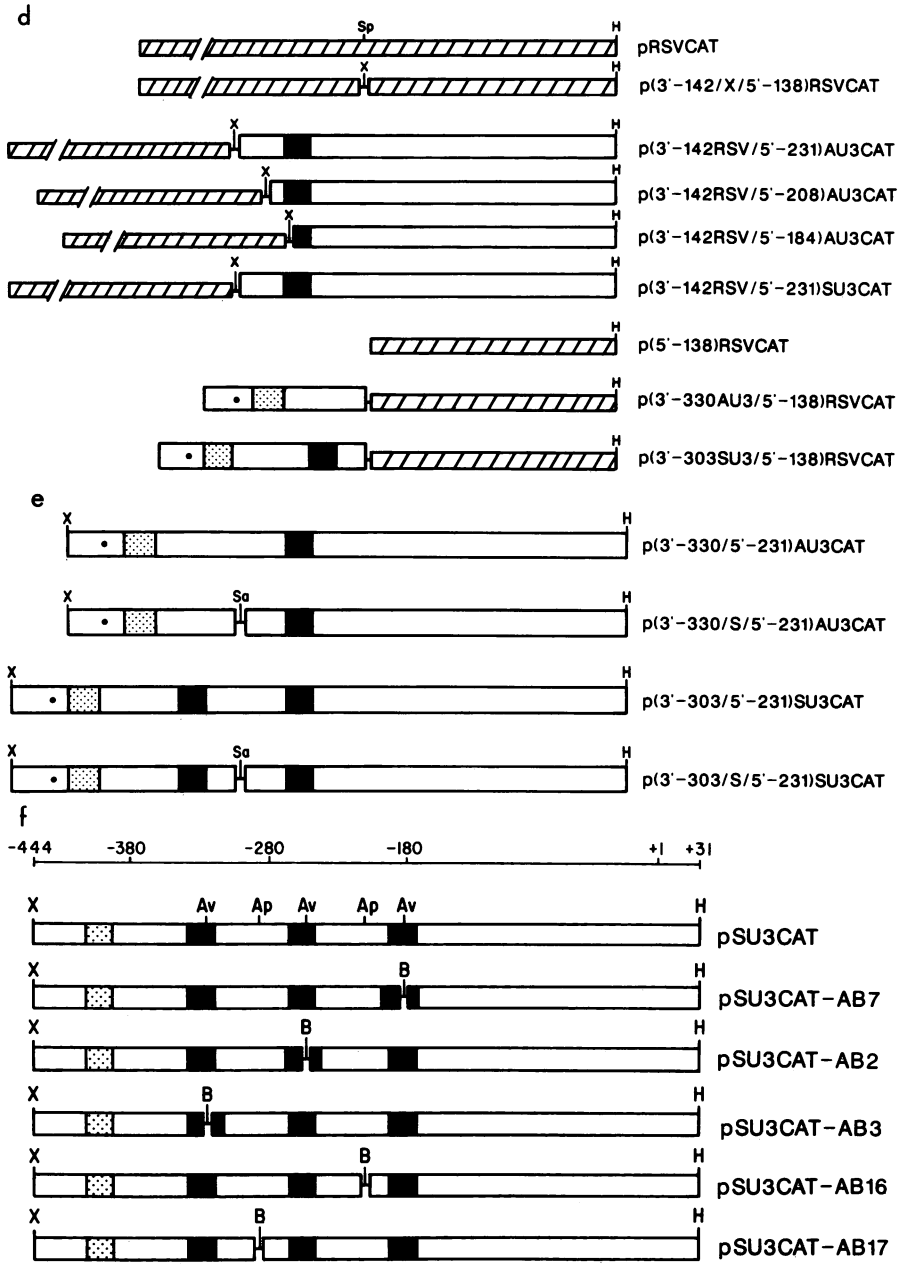



FIG. 2. Recombinant plasmids used in this study. (a) The locations of restriction enzyme sites in the Akv and SL3-3 repeat region are illustrated relative to the position of the consensus glucocorticoid sequences (▨, ■). The restriction enzyme sites illustrated are *Apa*I (Ap), *Ava*II (Av), *Eco*RV (RV), *Xho*I (X), and *Hin*dIII (H). (b) The internal deletions of the Akv repeat region are illustrated with their designations. (c) The internal deletions of the SL3-3 repeat region are illustrated with their designations. (d) The organization of the enhancer and promoter elements within the RSV 3' LTR is illustrated. The plasmid p(3'-142/X/5'-138)RSVCAT, which contains an *Xho*I site (X) in place of the natural *Sph*I restriction site present in the RSV LTR, is shown. The RSV promoter element that lacks an active enhancer [p(5'−138)RSVCAT] is also shown. The reciprocal recombinant plasmids that contain the RSV and murine LTR enhancer and promoter elements are shown. Symbols:

the transfected cultures were harvested. The amount of plasmid DNA used in the transfection experiments and the final concentration of dexamethasone used in treated-cell cultures are within the linear range of expressed CAT enzyme activity (4). The plasmids were transfected on the same day; often, more than one preparation of plasmid DNA for each constructed plasmid was used in transfection experiments.

**CAT enzyme assays.** Cell extracts were prepared by three cycles of freeze (−70°C)-thaw (+37°C) lysis of cell pellets suspended in 0.25 M Tris hydrochloride (pH 8.0). The cell extracts were clarified by centrifugation, and the cytoplasmic portions were used in the enzyme activity assays as described previously (3, 4, 14). Approximately 400 to 600 μg of total protein extract was used in each CAT assay. Data quantitation from the assay was done as described previously (3, 14). Linear time course experiments were conducted for sufficient lengths of time to generate measurable and quantitative data for all transfected plasmids. The mean slope values of enzymatic activity were calculated and




▨, RSV transcription elements; □, murine elements; ▩, ■, glucocorticoid regulatory consensus sequences as indicated in panel a. (e) The location of a synthetic 8-nucleotide *Sal*I (designated Sa) restriction linker in p(3'-330/5'-231)AU3CAT and p(3'-303/5'-231)SU3CAT is illustrated. (f) The location of a synthetic 8-nucleotide *Bgl*II (designated B) restriction linker in pSU3CAT at positions −184 (AB7), −208 (AB16), −256 (AB2), −280 (AB17), and −328 (AB3) is shown. In panels a to f, all transcription elements are linked to a CAT gene transcription unit (CAT coding sequences followed by the simian virus 40 small t-antigen intron and simian virus 40 early-region 3' processing and polyadenylation signals) 3' to the *Hin*dIII site shown.

expressed relative to pRSVCAT. The deviation about these values was 30% for basal-level expression and less than 20% for hormone-induced expression level of the murine LTR templates. Data from at least three experiments done on different days were found to be reproducible within these error ranges.

## RESULTS

The Akv LTR contains two types of sequences in the tandem repeat region that resemble consensus glucocorticoid response elements (GREs) (5). A 17-nucleotide sequence that shares extensive homology with an extended consensus GRE sequence (18) is located in the 3' portion of each 99-nucleotide repeat sequence (Fig. 1). The second element is a hexanucleotide core consensus GRE sequence (31, 37) present as a dyad symmetry 5' to the Akv repeat region (Fig. 1).

The role of these elements in the dexamethasone response of the Akv LTR was explored by deletion analysis. Small deletions were introduced into the viral LTR that was fused to the CAT gene. Plasmids carrying the modified LTRs were transfected into HeLA cells. The effect of the deletions on

the level of CAT activity in the presence and absence of dexamethasone was measured at doses of the drug previously shown to yield maximum stimulation of LTR activity (4). As shown previously (4), treatment of HeLa cells with 1 μM dexamethasone results in a sixfold increase in CAT enzyme activity directed by the plasmid that carries the complete Akv LTR fused to the CAT gene (pAU3CAT).

Removal of a single copy of the 99-nucleotide sequence (Fig. 2b, p(3'-330/5'-231)AU3CAT] resulted in a slight reduction of the constitutive expression of CAT activity. Only a slight reduction in the dexamethasone induction resulted from this deletion (Table 1). Removal of the majority of the remaining single copy of the remaining 99-nucleotide sequence as well as 5'-associated sequences [Fig. 2b, p(5'-231)AU3CAT or p(5'-208)AU3CAT] resulted in a further reduction in constitutive expression of CAT activity (Table 1). The level of CAT expression, however, was still increased by treatment of the HeLa cell recipients with dexamethasone (Table 1). Removal of an additional 24 base pairs [Fig. 2b, p(5'-184)AU3CAT] resulted in a threefold increase in constitutive expression of CAT activity. However, no increase in the level of CAT expression directed by this plasmid was observed in the presence of dexamethasone (Table 1).

To determine whether the decrease in the constitutive level of LTR activity could be uncoupled from the dexamethasone response, an enhancer from RSV was placed 5' to the deletions described above. Two such hybrid LTR elements [Fig. 2d, p(3'-142RSV/5'-231)AU3CAT and p(3'-142RSV/5'-208)AU3CAT] displayed greater constitutive CAT activity than pAU3CAT did. However, the glucocorticoid induction ratios directed by these LTR hybrids were similar to those of the mutant murine plasmids (Table 2). The third LTR hybrid [p(3'-142RSV/5'-184)AU3CAT] possessed constitutive levels threefold higher than those of the two other LTR hybrid mutants (Table 2). Unlike the other two LTR hybrids, however, the level of CAT expression directed by the third LTR hybrid was not increased by dexamethasone treatment. These experiments demonstrate that the ability of the murine plasmids to respond to dexamethasone is dependent upon specific sequences located in the Akv tandem repeat region. Furthermore, these sequences confer hormone-regulatory potential independent of the level of constitutive expression.

The inability to restore full induction potential of the murine transcription elements in the context of the RSV-Akv LTR hybrids prompted the examination of whether sequences present in the Akv enhancer region upstream of −330 are involved in the response to glucocorticoids. For this purpose, a hybrid promoter comprising a portion of the Akv enhancer linked to a RSV promoter [p(3'-330AU3/5'-138)RSVCAT] was constructed (Fig. 2d). The response of this hybrid promoter to dexamethasone was compared with that of the parental promoter element that lacks an enhancer [p(5'-138)RSVCAT] in HeLa cells. The sequences present in the Akv enhancer fragment stimulate CAT enzyme activity over threefold when linked to a heterologous promoter in the absence of dexamethasone. However, no increase in CAT expression as directed by this hybrid LTR was detected in the HeLa cell recipients treated with dexamethasone (Table 3). This result implies that the core consensus dyad symmetry 5' of the Akv repeat region is not sufficient to confer a response to glucocorticoids to a heterologous promoter.

**Localization of the response elements within the SL3-3 enhancer region.** The response of the LTR of the SL3-3 virus to dexamethasone is more pronounced than that of the Akv LTR. The level of CAT expression is increased approximately 25-fold by the steroid treatment (4). The large induction ratio can be attributed to the low level of CAT expression directed by the SL3-3 LTR in untreated HeLa cell recipients and the equivalence of the high level of CAT expression directed by the Akv and SL3-3 LTRs in the presence of dexamethasone. The GREs present in the SL3-3 enhancer region differ in two respects from those present in Akv (Fig. 1). First, the GRE is present in three copies in the SL3-3 repeat region, compared with two copies in Akv. Second, the location and sequence context of the GREs differ between SL3-3 and Akv. Like that of Akv, a core consensus element is present as a dyad symmetry 5' to the SL3-3 repeat region.

To define sequences important for high-level induction of the SL3-3 enhancer region in HeLa cells, we constructed a set of deletion mutants containing SL3-3 LTR sequences present in pSU3CAT. Removal of a single copy of the

TABLE 1. Sequences involved in glucocortoid regulation of the Akv LTR in HeLa cells

| Plasmid | In vitro CAT activity (relative to pRSVCAT)[a] | | |
|---|---|---|---|
| | −DEX[b] | +DEX | +/− ratio |
| pAU3CAT | 0.15 | 0.80 | 5.3 |
| p(3'-330/5'-231)AU3CAT | 0.12 | 0.50 | 4.2 |
| p(5'-231)AU3CAT | 0.04 | 0.12 | 3.0 |
| p(5'-208)AU3CAT | 0.02 | 0.06 | 3.0 |
| p(5'-184)AU3CAT | 0.06 | 0.06 | 1.0 |

[a] The absolute activity of pRSVCAT in HeLA cell transfection experiments described here is 0.9% acetylation of chloramphenicol per min in timed reactions with mixtures containing 400 μg of extract protein. The values reflected here represent mean slope values of enzymatic activity relative to pRSVCAT. The deviation about these values was 30% for basal-level expression and less than 20% for the hormone-induced expression level of the murine LTR templates. The plasmids shown in this table were transfected on the same day; data from at least three experiments done on different days were found to be reproducible within these error ranges.
[b] DEX, Dexamethasone.

TABLE 2. Effect of RSV enhancer upon glucocorticoid induction of murine LTRs in HeLa cells

| Plasmid | In vitro CAT activity (relative to pRSVCAT)[a] | | |
|---|---|---|---|
| | −DEX[b] | +DEX | +/− ratio |
| p(3'-142RSV/5'-231)AU3CAT | 0.14 | 0.42 | 3.0 |
| p(3'-142RSV/5'-208)AU3CAT | 0.20 | 0.70 | 3.5 |
| p(3'-142RSV/5'-184)AU3CAT | 0.58 | 0.82 | 1.4 |
| p(3'-142RSV/5'-231)SU3CAT | 0.12 | 0.40 | 3.3 |

[a] See Table 1, footnote *a*.
[b] DEX, Dexamethasone.

TABLE 3. Effect of murine enhancers upon glucocorticoid induction of the RSV LTR in HeLa cells

| Plasmid | In vitro CAT activity (relative to pRSVCAT)[a] | | |
|---|---|---|---|
| | −DEX[b] | +DEX | +/− ratio |
| p(5'-138)RSVCAT | 0.15 | 0.17 | 1.1 |
| p(3'-330AU3/5'-138)RSVCAT | 0.50 | 0.55 | 1.1 |
| p(3'-303SU3/5'-138)RSVCAT | 0.17 | 0.75 | 4.5 |

[a] See Table 1, footnote *a*.
[b] DEX, Dexamethasone.

72-nucleotide sequence [Fig. 2c, p(3'-303/5'-231)SU3CAT] resulted in no significant change in the constitutive-level or in the high-level induction of CAT expression in response to dexamethasone (Table 4). Removal of the majority of the remaining single copy of the 72-nucleotide sequence as well as 5'- associated sequences [Fig. 2c, p(5'-231)SU3CAT] resulted in a slight reduction in the constitutive activity. However, the induction of CAT expression from this deletion mutant was reduced to 15% of the wild-type levels observed with pSU3CAT (Table 4). This mutant also displays an induction ratio comparable to that of similar Akv LTR constructs. Since the nucleotide sequences are identical for Akv and SL3-3 3' to nucleotide position −225, the corresponding deletion endpoints at nucleotide positions −208 and −184 were not constructed or analyzed with SL3-3-LTR templates. However, a comparison of p(5'-231)SU3CAT with p(5'-208)AU3CAT indicates that p(5'-231)SU3CAT displays twofold-higher constitutive activity without a change in induction potential (Tables 1 and 4). As was observed for the Akv LTR, addition of an RSV enhancer 5' to the deleted SL3-3 LTR (p3'-142RSV/5'-231)SU3CAT increased the constitutive activity of the LTR but did not significantly alter the dexamethasone induction ratio (Table 2). An additional plasmid was made in which the 5' portion of the SL3-3 LTR that contains one of the GRE sequence was placed 5' to the RSV promoter (Fig. 2d). No significant increase in the constitutive activity of the RSV promoter was observed. However, in contrast to the RSV promoter alone, treatment with dexamethasone of this modified plasmid resulted in a 4.5-fold increase in CAT activity (Table 3).

Although the constitutive activity of the SL3-3 LTR is low, approximately that of the Akv LTR deleted for all but one of the GRE sequences [p(5'-208)AU3CAT], the activity was increased very markedly by the presence of dexamethasone to a level that is similar to that of the intact Akv LTR in the presence of the steroid. The induced activity level is about 8 to 10 times that observed for the plasmids that carried the deleted Akv LTR, p(5'-231)AU3CAT and p(5'-208)AU3CAT. This observation implies that sequences within the tandem repeat region of the SL3-3 LTR confer a heightened response to dexamethasone. These sequences must be contained within the region remaining in the SL3-3 LTR deleted for one of the 72-nucleotide repeats, since the plasmid p(3'-303/5'-231)SU3CAT displays an activity similar to that of the pSU3CAT plasmid in the presence or absence of dexamethasone.

TABLE 4. Sequences involved in glucocorticoid regulation of the SL3-3 LTR in HeLa cells

| Plasmid | In vitro CAT activity (relative to pRSVCAT)[a] | | |
|---|---|---|---|
| | −DEX[b] | +DEX | +/− ratio |
| pSU3CAT | 0.03 | 0.80 | 26.0 |
| p(3'-303/5'-231)SU3CAT | 0.04 | 0.80 | 20.0 |
| p(5'-231)SU3CAT | 0.02 | 0.06 | 3.0 |
| p(3'-303/5'-208)SU3CAT | 0.02 | 0.28 | 14.0 |
| p(3'-303/5'-184)SU3CAT | 0.05 | 0.12 | 2.4 |
| p(3'-312/5'-208)SU3CAT | 0.02 | 0.50 | 25.0 |
| p(3'-326/5'-208)SU3CAT | 0.03 | 0.41 | 13.5 |
| p(3'-333/5'-208)SU3CAT | 0.02 | 0.30 | 15.0 |
| p(3'-335/5'-208)SU3CAT | 0.02 | 0.11 | 5.5 |
| p(3'-336/5'-208)SU3CAT | 0.02 | 0.09 | 4.5 |
| p(3'-341/5'-208)SU3CAT | 0.01 | 0.09 | 9.0 |
| p(3'-353/5'-208)SU3CAT | 0.01 | 0.05 | 5.0 |

[a] See Table 1, footnote *a*.
[b] DEX, Dexamethasone.

TABLE 5. Effect of inserting extra sequences into murine enhancers upon glucocorticoid induction of murine LTRs in HeLa cells

| Plasmid | In vitro CAT activity (relative to pRSVCAT)[a] | | |
|---|---|---|---|
| | −DEX[b] | +DEX | +/− ratio |
| p(3'-330/5'-231)AU3CAT | 0.12 | 0.50 | 4.2 |
| p(3'-330/S/5'-231)AU3CAT | 0.12 | 0.50 | 4.2 |
| p(3'-303/5'-231)SU3CAT | 0.04 | 0.80 | 20.0 |
| p(3'-303/S/5'-231)SU3CAT | 0.04 | 0.23 | 5.7 |

[a] See Table 1, footnote *a*.
[b] DEX, Dexamethasone.

To examine what sequences were necessary for high-level induction, we introduced a series of deletions starting at either an *Eco*RV or an *Apa*I restriction site located between the GRE sequences present on the plasmid p(3'-303/5'-231)SU3CAT (Fig. 2c). Deletion of sequences between the GRE elements reduced the activity of the LTRs in the presence of dexamethasone. The level was reduced to 35% and 15% of that of the starting plasmid by deletions present on plasmids p(3'-303/5'-208)SU3CAT and p(3'-303/5'-184)SU3CAT, respectively (Table 4). Extension of deletion of the latter plasmid in the 5' direction to nucleotides −312, −326, −333, −335, and −336 resulted in reduction of the induced level of CAT activity by 38%, 49%, 62%, 86%, and 89%, respectively (Table 4). Further deletion in the 5' direction to nucleotide −341 did not result in a further decrease in the activity of CAT enzyme in the presence of dexamethasone, whereas an additional deletion of −353 resulted in a 94% reduction in the induced level of enzyme activity (Table 4). These results indicate that the 5' portions of both GRE sequences are required for high induction ratios. They also show that the sequences that lie between the two GRE elements contribute to the high level of activity in the presence of dexamethasone.

The importance of sequence composition in the region that lies between the two GRE elements of the p(3'-303/5'-231)SU3CAT plasmid for high-level dexamethasone induction was explored by insertion of an 8-nucleotide *Sal*I restriction site linker (Fig. 2e). Insertion of this linker resulted in a fourfold reduction in the level of CAT activity synthesized in the presence of dexamethasone (Table 5). The constitutive activity of the plasmid p(3'-303/S/5'-231)SU3CAT was unaffected by this insertion (Table 5). Insertion of the same 8-nucleotide sequence at the same position relative to the promoter-proximal GRE sequence of plasmid p(3'-330/5'-231)AU3CAT (Fig. 2e) did not result in a measurable change in the constitutive or induced activity of this LTR (Table 5). These results imply that the 8-nucleotide sequence does not itself encode a regulatory function.

The striking effect of these mutations within derivatives of the SL3-3 LTR prompted us to examine the effect upon dexamethasone induction of inserting synthetic linkers into various locations in the entire, intact SL3-3 repeat region present in pSU3CAT. An 8-nucleotide synthetic *Bgl*II site linker was inserted into each of the two *Apa*I sites that lie between adjacent GRE sequences, as well as into each of the three *Ava*II sites present internal to the GRE sequence (Fig. 2f). Insertion of a linker into the most distal GRE sequence or between the two most distal GRE sequences has no effect upon dexamethasone stimulation of the SL3-3 LTR (Fig. 3,

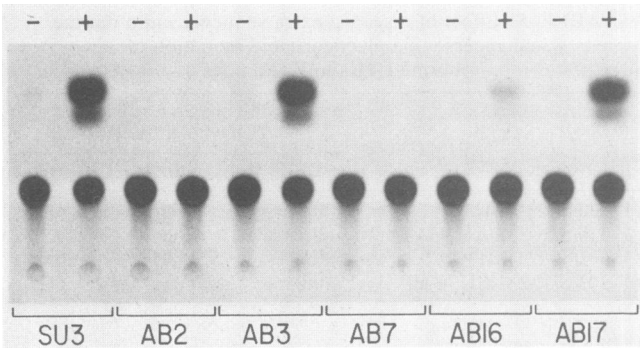



FIG. 3. Representative CAT assay data demonstrating the effect of linker insertions into the SL3-3 LTR of pSU3CAT. The designations refer to the mutant LTR plasmid used in the transfection and correspond to wild-type SL3-3 LTR (SU3), or *Bgl*II linker insertions in the SL3-3 LTR at positions −256 (AB2), −328 (AB3), −184 (AB7), −208 (AB16), and −280 (AB17). The CAT enzyme activity of extracts prepared from transfected recipient cells untreated (−) or treated (+) with 1 μM dexamethasone is illustrated. The autoradiogram of the reaction mixtures resolved by thin-layer chromatography is shown, in which the lower spot corresponds to the substrate, chloramphenicol, and the upper two spots correspond to the acetylated product forms. Linear time course experiments were performed; a 40-min time point is shown. The data shown here are from a representative experiment that has been done on three different occasions, with similar results obtained each time.

lanes AB3 and AB17). However, insertion of the synthetic linker between the proximal and first-distal GRE sequences reduced high-level induction (Fig. 3, lanes AB16), whereas insertion of the linker into either of these two sequences abolished the response to dexamethasone (Fig. 3, lanes AB2 and AB7).

## DISCUSSION

Both Akv and SL3-3 U3 regions are responsive to dexamethasone in HeLa cells. This response was shown previously to be mediated by sequences in the tandem repeat region of each viral LTR (4). The experiments presented here located the sequences necessary for the observed dexamethasone responsiveness. These sequences share extensive homology with previously identified elements that respond to glucocorticoids in vivo (2, 5, 9, 17, 18, 20, 25, 29, 30, 34) and contain the hexanucleotide sequence that is protected by the glucocorticoid receptor complex in vitro (5, 9, 18, 29, 31, 37, 38, 47).

Specific insertions of extra nucleotides into the GRE sequences of the SL3-3 LTR revealed two novel aspects of the hormone-regulatory properties of this LTR. Although these GRE sequences reside in redundant sequence information, they do not behave identically in specifying the SL3-3 LTR response to dexamethasone: the integrity and sequence context of the two promoter-proximal copies of the GRE sequence are critical for the hormone-responsive nature of this LTR. This may explain why removal of one copy of the 72-base-pair repeat monomer in SL3-3 does not dramatically alter the response of the LTR to dexamethasone administration. The second-distal GRE (and associated) sequence(s) positionally replaces the deleted first-distal GRE sequence, and high-level inducibility is maintained. The SL3-3 LTR plasmids that contain a linker insertion at a single GRE sequence have different phenotypes from isolated, intact GRE sequences. On the basis of data presented for isolated GRE sequences, we expected to observe some inducibility from all three SL3-3 LTR mutant plasmids (AB2, AB3, and AB7), since at least two intact GRE sequences are present in each of these plasmids. However, two of these plasmids (AB2 and AB7) failed to respond to dexamethasone treatment. We conclude that although single elements can behave as discrete inducible enhancers, the regulation of the multiple-element response must be extraordinarily complex.

The induction profile of two response elements present in their natural context is greater than the sum of the induction profile for two separate elements. These observations suggest that the natural organization of the SL3-3 tandem repeat region may permit interactions between the effector molecules. Such a model predicts that spatial relationships of the SL3-3 response elements are important for efficient regulation. In this regard, two controlling elements that contain the same number of response elements do not display similar induction profiles [pAU3CAT and p(3′-303/5′-231]. On the basis of the data presented for the effect of linker insertions in p(3′-303/5′-231)SU3CAT and pSU3CAT, the spacing between the two promoter-proximal GRE sequences has a great impact upon inducibility of this LTR. The relationship of the distal GRE, as well as the 5′ dyad symmetry element, to the two promoter-proximal GRE sequences does not appear critical in specifying high-level inducibility of pSU3CAT. The organization of the GRE sequences adjacent to the viral promoter appears to dictate the overall response profile of the promoter.

The tandem repeat regions of Akv and SL3-3 also differ with respect to sequence organization surrounding the GRE sequences, and we expect that the observed functional differences between these two enhancers are also specified by their sequence organization (3, 4). The equivalence of high-level activity of Akv and SL3-3 enhancer regions in the presence of the steroid could be explained by rate-limiting formation of a transcription complex at the promoter site (8). The recruitment of factors required for active promoter utilization is probably specified by sequences within the enhancer element (8, 12, 43, 46). In vitro analysis of the related Moloney murine leukemia virus tandem repeat region revealed that multiple factors present in nuclear extracts recognize adjacent and overlapping sequences within this enhancer element (41). The availability of such factors to bind to the template will depend upon the organization of the cognate binding sites with respect to other sequence elements, as well as interactions with other DNA-binding proteins already present. The results of the sequence deletions and insertions upon the SL3-3 LTR hormone responsiveness argue that such mutations removed information involved in either the organization of the SL3-3 repeat region for efficient DNA-hormone receptor complex formation or the recruitment of additional factors to the template after DNA-hormone receptor complex formation occurs.

The organization of discrete regulatory sequences plays an important role in the hormone-related expression of these transcription elements in one cellular context. The sequences present within the Akv and SL3-3 enhancer regions are organized in a modular arrangement (21). At least part of the differential constitutive activity of these repeat regions in different cell types is attributed to this modular organization (3, 4). Modular organization of enhancers has been proposed also for the role of short-sequence duplications in RSV (19), polyomavirus (33, 36, 44), simian virus 40 (15, 50), and immunoglobin heavy-chain (45) enhancer function within particular cell types. Although regulation of cell type preference of enhancers could be achieved by interaction of cell type-specific factors with unique sequences (28, 39, 41), this

probably is not the only mechanism. Cell-specific regulation could also be accomplished through the alternative interactions of adjacent sequences with factors common to many cell types (9, 39, 41, 48). Modular sequence organization may provide a means whereby enhancer activity is regulated in a particular cellular context.

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