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A Complex Androgen-Responsive Enhancer Resides 2 Kilobases Upstream of the Mouse Slp Gene

FABRIZIO LORENI, JEFFREY STAVENHAGEN, MARTHA KALFF, AND DIANE M. ROBINS*
Department of Biological Sciences, Columbia University, New York, New York 10027

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Neighboring genes encoding the mouse sex-limited protein (Slp) and fourth component of complement (C4) show extensive homology. In contrast to C4, however, Slp is regulated by androgen. One region of the Slp gene capable of hormonal response following transfection was located about 2 kilobases upstream of the transcription start site, where the C4 and Slp sequences diverge. This region, delimited here to a 0.75-kilobase fragment, showed cryptic promoter activity as well as androgen responsiveness in either orientation in front of the bacterial chloramphenicol acetyltransferase coding region. When this fragment was placed upstream of a viral long terminal repeat, increased chloramphenicol acetyltransferase expression derived from the viral promoter. Proteins from nuclear extracts specifically bound to four sequences within the region, near sites that are DNase I hypersensitive in vivo and reflect the hormonal and developmental regulation of Slp. Like several other cellular enhancers, this androgen-responsive element seems to be modular in nature and complex in its function.

Regulation of steroid hormone-inducible genes is one of the few instances in higher eucaryotes for which small-molecule inducers of gene expression, and the factors with which they interact, are biochemically known. Steroid hormones bind to intracellular receptor proteins that then bind specific DNA sequences of responsive genes to modulate transcription (39). These sequences function as transcriptional enhancer elements in that their effect on their own or heterologous promoters does not depend stringently on distance or orientation. In vivo, the expression of responsive genes is often complex due to the action of other hormones and tissue-specific factors, which also may work via enhancerlike sequences (4, 8, 12). In addition, steroid hormones can affect transcription of specific genes indirectly (1) and can affect posttranscriptional processes, such as RNA stabilization (2).

We have been studying the mouse sex-limited protein (Slp) gene, which is under hormonal, developmental, and tissue-specific control (33). Slp arose from a tandemly duplicated complement component C4 gene within the mouse major histocompatibility complex (*H-2*) (5). Slp differs from C4 in its inability to participate in the complement cascade and in its regulation by androgen. Both serum proteins are synthesized at moderate levels in the liver, and C4 is synthesized at high levels in peritoneal macrophages (33). The androgen dependence of Slp is evidenced by its expression in mature male mice at 100-fold-higher levels than in females (10). Despite functional and regulatory differences, C4 and Slp sequences diverge by less than 4%, both in their coding regions (26) and in about 2 kilobases (kb) of upstream flanking DNA (23); homology is maintained for over 55 kb encompassing the genes (5). This sequence similarity, coupled with the fact that there are alleles of both genes that differ in expression, allows correlation of divergent DNA sequences with variations in gene regulation.

C4 and Slp DNA clones from several mouse strains have been characterized (14, 24, 26). In strains with *H-2*-linked androgen-independent Slp expression, such as B10.WR, multiple Slp genes exist in the genome; apparent recombination with C4 in 5′ regions is likely to account for their C4-like regulation (22, 29, 35). One of four B10.WR Slp genes, however, is more similar to the single androgen-dependent allele from B10.D2 mice; furthermore, an upstream region present in both of these Slp genes directed androgen-responsive transcription after transfection (35). In liver chromatin, hormone-responsive DNase-hypersensitive sites are located in the same upstream region, 2 kb from the Slp transcription start site (15), confirming its significance to expression in vivo. This region is beyond the breakpoint in C4-Slp homology that occurs at −1.9 kb (23).

In this report we characterize further the Slp upstream region. In transfection, a 750-base-pair (bp) fragment was sufficient, in either orientation, for androgen-inducible expression of chloramphenicol acetyltransferase (CAT). Cryptic promoters within the fragment were not evident in vivo but were revealed in the context of various gene fusions. When placed upstream of the Rous sarcoma virus long terminal repeat (LTR), the fragment activated transcription from the strong viral promoter. Sequences within this region bound proteins specifically and may provide a basis for functional dissection of this complex hormone-responsive enhancer.

MATERIALS AND METHODS

Construction of *cat* fusion plasmids. The pG3CATF vector and plasmids with the promoter and flanking *Bam*HI fragments of Slp and C4 (see Fig. 2) have been described (35). Deletions of the Slp fragment were constructed as follows (see Fig. 4). For pG2.9RΔ5, the 0.75-kb *Ava*I-*Xba*I fragment within the 2.9-kb *Bam*HI fragment of Slp was treated with Klenow fragment at the *Xba*I site and inserted into *Sma*I-*Ava*I-digested pG2.9R. The resulting plasmid, pG2.9RΔ5, has the 1.1-kb *Bam*HI-*Xba*I fragment (distal to the Slp promoter) in front of the *cat* gene in the reverse orientation. For pG2.9RΔ6, pG2.9RΔ5 was digested with *Sma*I and

* Corresponding author.

*Bam*HI, treated with Klenow, and religated, leaving the 0.75-kb *Sma*I-*Xba*I fragment before *cat* in the reverse orientation. For pG2.9Δ9, the 0.75-kb *Sma*I-*Xba*I fragment was obtained in the natural orientation in front of *cat* by inserting the fragment into *Sma*I-*Xba*I-digested pG3CATF. For pG2.9Δ6, pG2.9 was digested with *Sma*I, partially digested with *Sca*I, and religated. The construct isolated contains the 0.25-kb *Sca*I-*Bam*HI fragment encompassing the Slp promoter in the natural orientation before *cat*. For pG2.9Δ8A and pG2.9Δ8B, the 0.75-kb *Sma*I-*Xba*I fragment was treated with Klenow and inserted in pG2.9 that had been digested with *Sma*I and partially digested with *Sca*I. Plasmids were isolated that had the *Sma*I-*Xba*I fragment in the natural orientation before the Slp promoter (pG2.9Δ8A) and in the opposite orientation (pG2.9Δ8B). For pA2.9B and pA2.9RB, the Slp 2.9-kb *Bam*HI fragment was inserted in the BamHI site 3′ to the *cat* gene in the vector $pA_{10}CAT2$ (17). The Slp promoter is in the same orientation as the simian virus 40 (SV40) promoter in pA2.9B and opposite it in pA2.9RB (see Fig. 6A). For pG3CRL, the 0.2-kb *Hin*dIII fragment from pJA150 (16), containing the Rous sarcoma virus (RSV) transcription start site and about 50 bp of untranslated region, was inserted in the *Hin*dIII site 5′ to the *cat* gene in pG3CATF. The resulting vector, pG3CRL, has the *cat* gene driven by the RSV LTR promoter. For pGΔ9CRL and pGRΔ6CRL, the 0.75-kb *Sac*I-*Xba*I fragments from plasmids pG2.9Δ9 and pG2.9RΔ6 were inserted in *Sac*I-*Xba*I-digested pG3CRL. The resulting plasmids contained the original 0.75-kb *Sma*I-*Xba*I fragment from the Slp 5′-flanking region in the natural orientation (pGΔ9CRL) and reversed (pGRΔ6CRL) with respect to the LTR and the *cat* gene.

Cells and transfection. T47D cells were grown in Dulbecco modified Eagle medium with 10% fetal calf serum and 0.6 μg of insulin per ml. G221 cells were grown in Dulbecco modified Eagle medium with 10% fetal calf serum and 10 ng of dihydrotestosterone (DHT) per ml (3×10^{-8} M). Ltk^- cells were maintained as described (38). G221 cells were transfected by the $CaPO_4$ method (38) as detailed previously (35), and T47D cells were transfected by the DEAE-dextran method (4). For androgen response, G221 cells were withdrawn from DHT for 48 h; T47D cells were treated for 48 h with 1 μg of DHT per ml. For RNA analysis of transiently transfected T47D cells, cells were plated at 5×10^6 per 150-mm dish and transfected with 25 μg of plasmid DNA.

CAT assays. CAT activity of cell extracts was assayed as described (11) with the following modifications. Cell extracts were made 44 to 48 h after transient transfection or after hormone withdrawal of stable G221 transformants by five freeze-thaw cycles. Protein concentration was determined by the Bradford method (Bio-Rad). For transient transfections, samples containing 50 to 100 μg of protein were incubated in 200 μl of 0.25 M Tris (pH 7.5)–0.4 μM acetyl coenzyme A–0.2 μCi of [^{14}C]chloramphenicol for 2 h at 37°C. In experiments with weakly expressing constructs, samples were also incubated for 16 h with 2 mM acetyl coenzyme A and 0.5 μCi of [^{14}C]chloramphenicol. For the stable transformants, assays included only 5 to 15 μg of protein.

RNase mapping. Total cellular RNA was prepared by the guanidinium-LiCl method (3). Single-stranded RNA probes were synthesized and gel eluted, and RNase mapping was performed as described (41) with 20 μg of RNase A and 1 μg of RNase T_1 per ml, unless noted otherwise.

Preparation of nuclear extracts. Fresh and frozen tissues gave similar results. Fresh mouse livers were minced on ice; frozen livers were ground to a powder under liquid nitrogen with a mortar and pestle. Cultured cells were harvested in Dulbecco phosphate-buffered saline and collected by centrifugation. Tissues and cells were washed with ice-cold phosphate-buffered saline, suspended in 10 volumes of HB (0.25 M sucrose, 25 mM KCl, 3 mM $MgCl_2$, 50 mM Tris hydrochloride [pH 7.5], 1 mM phenylmethylsulfonyl fluoride), and homogenized with a loose pestle until at least 90% of the cells were lysed, as judged by microscopy. Nuclei were pelleted in a clinical centrifuge for 10 min, and extracts were prepared as described (27).

DNase I footprint analysis. Footprint reactions were done as described (27) with the following modifications. Extracts were preincubated for 20 min at 25°C with 2 μg of poly[d(I-C)] as a nonspecific competitor. From 0.5 to 1.0 ng of end-labeled fragment (2.5×10^4 cpm) was then added, and incubation was continued for 40 min. DNase I digestion was carried out at 0°C for 5 min with 1 to 3 μl of a freshly diluted 250-μg/μl enzyme suspension.

DNA sequence analysis. Restriction fragments were subcloned into M13 vectors (21), and both strands were sequenced by the chain termination method (30).

RESULTS

A transfected full-length Slp gene is hormone responsive. To determine whether hormonal response could be achieved following transfection, an entire Slp gene was tested. The gene was reconstructed from phage clones of a B10.D2 genomic library (15). Two overlapping phage inserts were joined in a plasmid vector to establish an intact 16-kb structural gene with 11 kb of flanking information upstream and 3 kb downstream. An intact C4 gene was derived similarly. The G221 subline of mouse Shionogii S115 mammary carcinoma cells was used in gene transfer, as it has characterized androgen receptors (34) and can regulate other androgen-responsive genes following transfection (28). Furthermore, these cells (see below) as well as mammary glands (Cox and Robins, manuscript submitted) appear to synthesize C4. While hepatocyte lines might be preferable, none are known that contain functional androgen receptor. The reconstructed Slp and C4 genes were donated to the G221 cells by using G418 resistance to select stable cell lines. Details of transformant expression will be presented elsewhere (Kalff and Robins, manuscript in preparation); an initial result is shown below as reference for subsequent experiments.

Several independent transformants containing exogenous Slp or C4 genes were screened for expression when grown in the presence of DHT or 48 h after hormone withdrawal. Slp cotransformants expressed severalfold more mRNA in the presence than in the absence of androgen, as seen on Northern (RNA) blots probed with cDNA that hybridizes to both C4 and Slp 5.4-kb mRNAs (Fig. 1). In contrast, the mRNA level in C4 transformants was unchanged by hormone (Kalff and Robins, in preparation). Cross-hybridizing RNA detected in the parent cell line was also unaffected by hormone.

That Slp mRNA levels respond to hormone in these cells indicates that within the 30-kb insert of the donated Slp plasmid reside sequences allowing gene regulation. The low level of expression and the endogenous transcripts made it difficult to determine the basis of the induction (i.e., direct or indirect, pre- or posttranscriptional). These complications

were circumvented by searching directly for sequences that would regulate a reporter gene.

A hormone-responsive element lies within 3 kb of the Slp promoter. DNase I-hypersensitive sites 2.0 and 2.3 kb upstream of Slp correlate with its hormonal regulation (15). Therefore, we tested this region for regulatory capacity after fusion to the bacterial *cat* gene. The *cat* coding cassette of pSV2CAT (11) without the SV40 promoter and enhancer was placed into pGem3 (to give pG3CATF), and *Bam*HI fragments spanning the transcription start sites of C4 and Slp were inserted (Fig. 2). These fragments contain about 50 bp of 5′ untranslated region and 2.9 kb (Slp) or 2.6 kb (C4) of 5′-flanking DNA.

For transient assay of fusion plasmids in hormone-responsive cells, a human mammary cell line, T47D (4), was used, as G221 cells used in stable transfection showed poor transient activity. After 48 h, the only significant CAT expression was with the construct containing the Slp promoter fragment in the reversed orientation (pG2.9R; Fig. 2). Furthermore, this activity was increased fivefold by androgen, attaining 25% of the pSV2CAT level. These results were surprising in that the promoters of both C4 (pG2.6) and Slp (pG2.9) failed to work, while the activity of pG2.9R suggested the presence of an upstream opposite-strand inducible promoter in the Slp fragment.

In an attempt to understand the lack of expression of C4 and Slp plasmids, they were stably transfected into G221 cells, which regulate the full Slp gene. Basal expression was obtained for Slp and C4 fragments correctly oriented in front of *cat*, but only the reverse Slp plasmid was hormone responsive. That is, cells with integrated copies of pG2.9 showed low levels of uninducible CAT activity, cells with pG2.6 showed about two- to fivefold that activity, and transformants with pG2.9R expressed high CAT levels that were threefold greater with androgen. Regulated expression

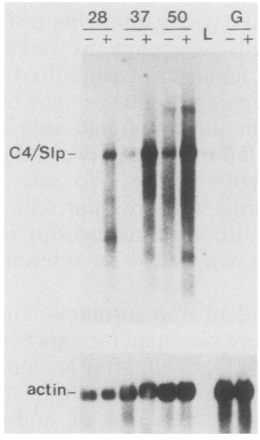

FIG. 1. Androgen regulation of the Slp gene in G221 cells. Polyadenylated [poly(A)$^+$] RNA from cells grown for 48 h in the absence (−) or presence (+) of DHT (10 ng/ml) was electrophoresed through 0.8% agarose–6% formaldehyde and transferred to GeneScreen Plus. RNA (10 μg) was loaded in each lane for transformants 37, 50, and G221 (G) cells; for clone 28, 5 μg of RNA was used. Lane L is 20 ng of poly(A)$^+$ B10.D2 liver RNA. The upper panel shows hybridization with nick-translated C4 cDNA; 5.4-kb C4/Slp mRNA is indicated. The filter was rehybridized with actin cDNA (lower panel) to show similar amounts of RNA in paired lanes (the L lane appears blank due to the small amount of RNA); autoradiographic exposure with this probe was for a few hours, compared with several days for C4.

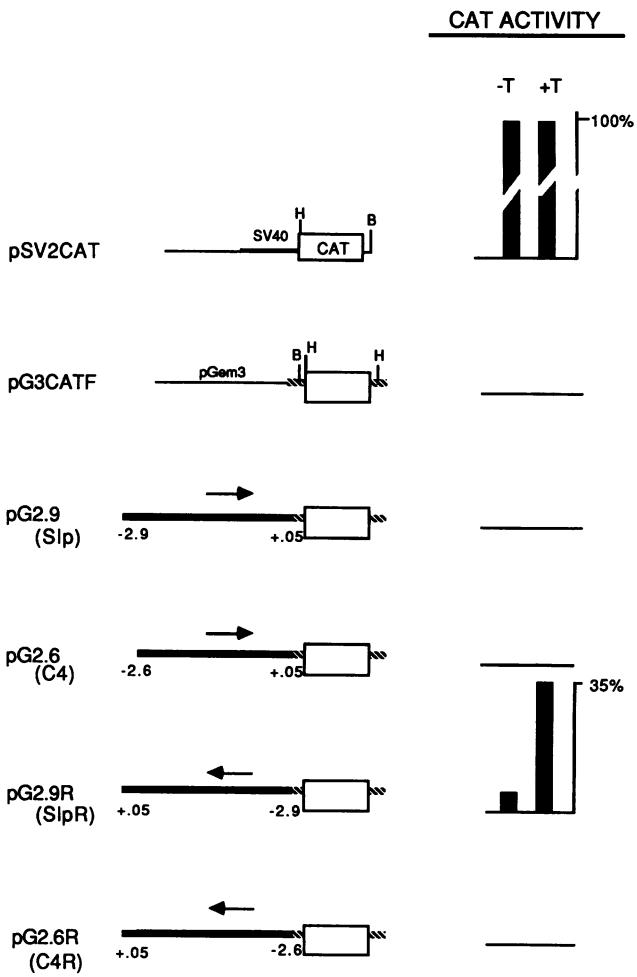

FIG. 2. CAT activities directed by 5′-flanking DNA fragments of C4 and Slp. pG3CATF was derived by placing the *cat* coding cassette of pSV2CAT into pGem3 (35). *Bam*HI (B) and *Hin*dIII (H) sites are shown; the polylinker of pGem3 appears stippled next to the *cat* gene. The T7 RNA polymerase promoter is before the *cat* gene and the SP6 promoter is downstream. *Bam*HI fragments encompassing C4 and Slp promoters were inserted in both orientations in pG3CATF. Positions (in kilobases) relative to C4/Slp cap sites are shown at the ends of the inserts; orientation relative to C4/Slp transcription is indicated by arrows. T47D cells were transiently transfected with these plasmids and grown in the absence (−T) or presence (+T) of DHT (1 μg/ml). CAT activities are relative to pSV2CAT activity averaged over four independent transfections.

of the full gene but not the *cat* construct with the correctly oriented Slp fragment may indicate the influence of sequences further upstream or downstream. In particular, these sequences might overcome a negative element described by Nonaka et al. (23) as residing 300 to 450 bp upstream of both C4 and Slp promoters. These authors found that in HepG2 liver cells, a negative effect on transcription was overcome for C4 by sequences farther upstream but only partially overcome by Slp upstream sequences. Thus, the hormone response shown by the Slp fragment reversed may be due to one of several elements involved in regulation of the Slp gene.

To characterize the upstream hormone-responsive region revealed in pG2.9R, transcription start sites were deter-

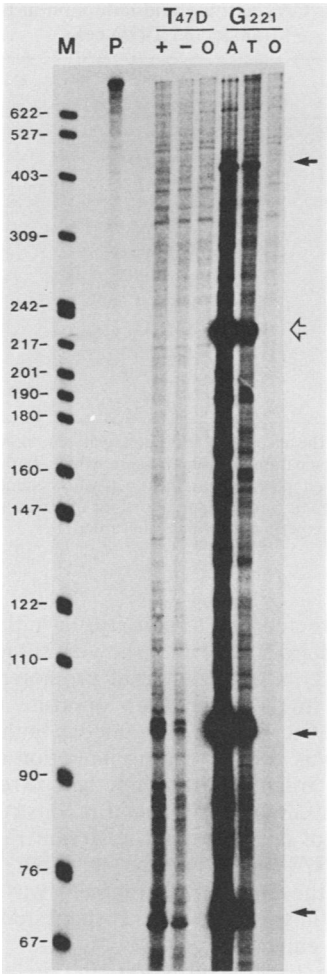

FIG. 3. Transcription initiation sites within the Slp upstream region. RNA from cells transfected with pG2.9R was mapped by using an 800-nucleotide (nt) probe synthesized with T7 polymerase from pG2.9Δ9 (see Fig. 4) digested with *Hin*dIII; this probe protects transcripts in the *Sma*I-*Xba*I region from the opposite strand as Slp transcripts. T47D cells were transfected and grown in the presence (+) or absence (−) of DHT (1 μg/ml) for 48 h in serum free of endogenous steroids. Total RNA (60 μg) from these cells and untransfected T47D cells (O) was treated with RNase-free DNase prior to mapping. RNA from stably transfected G221 cells was as follows: lane A, 2 μg of poly(A)$^+$ RNA; lane T, 10 μg of total RNA; lane O, probe incubated and digested without additional RNA; lane M, *Hpa*II-cleaved pBR322 DNA; lane P, probe. Black arrows indicate the major start sites 70, 105, and 420 bases from the *Sma*I site; the open arrow marks a 255-base internal RNA.

mined for RNA from transiently transfected T47D cells and stable G221 transformants (in which RNA levels are higher). Several prominent transcription start sites, detected by RNase mapping, were seen consistently in both cell lines (Fig. 3, bands of 70, 100 and 425 bases). These correspond to 470, 500, and 825 bases from the point of fusion to the *cat* gene, or about 2.1 and 2.4 kb upstream of the Slp cap site. In the presence of androgen, these transcripts were more abundant. Using additional probes, we found that the protected RNA at 225 bases in G221 cells was derived entirely from within this region. Which fusion mRNAs led to functional *cat* mRNA is unknown, but all would have extensive leader sequences before the normal *cat* translation start site. These transcripts are not detected in liver RNA (35); instead, the start site positions correspond strikingly to Slp's nuclease-sensitive sites.

To show that the response of the Slp upstream region to androgen was specific, the effect of other hormones was tested. In T47D cells, response was increased an additional twofold by raising androgen from physiological (10^{-8} M) to pharmacological (10^{-6} M) concentrations, while in G221 cells the response was maximal at 3×10^{-8} M, possibly due to different receptor levels of the two lines. Estrogen had no effect on CAT activity in either cell line, indicating that DHT response is not due to metabolic conversion or to cross-reaction with the estrogen receptor, as may occur with high androgen doses (40). Simultaneous administration of the antiandrogen flutamide decreased induction. The androgen response occurred (and was somewhat greater) in serum free of endogenous steroids, indicating no dependence on other steroids. An interesting finding (of unknown significance to in vivo regulation) was that induction by progesterone was greater than by testosterone in T47D cells; it was not decreased by flutamide and so did not work via the androgen receptor.

A 750-bp fragment is sufficient for androgen-regulated CAT

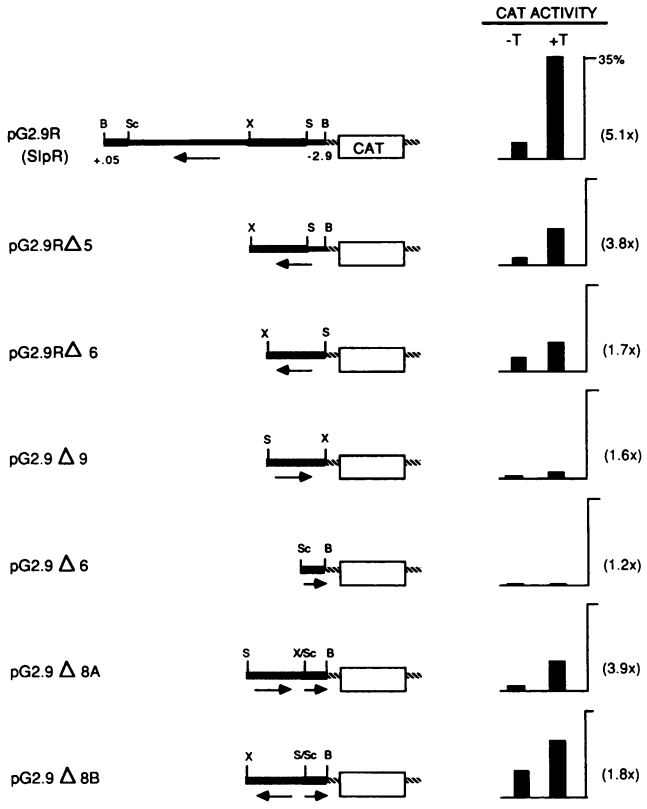

FIG. 4. CAT activity of fusion genes with deleted Slp upstream regions. Fusion plasmids were derived from pG2.9 and pG2.9R as described in the text. The stippled box represents the 750-bp *Sma*I-*Xba*I fragment upstream of the Slp promoter; the 250-bp *Sca*I-*Bam*HI fragment is represented by a black box. Arrows show the orientation of fragments with respect to Slp transcription. Restriction sites (B, *Bam*HI; Sc, *Sca*I; X, *Xba*I; S, *Sma*I) are shown for reference, as most sites were lost in cloning. CAT activity is shown as percentage of pSV2CAT activity averaged over four transfections. The mean induction is shown in parentheses.

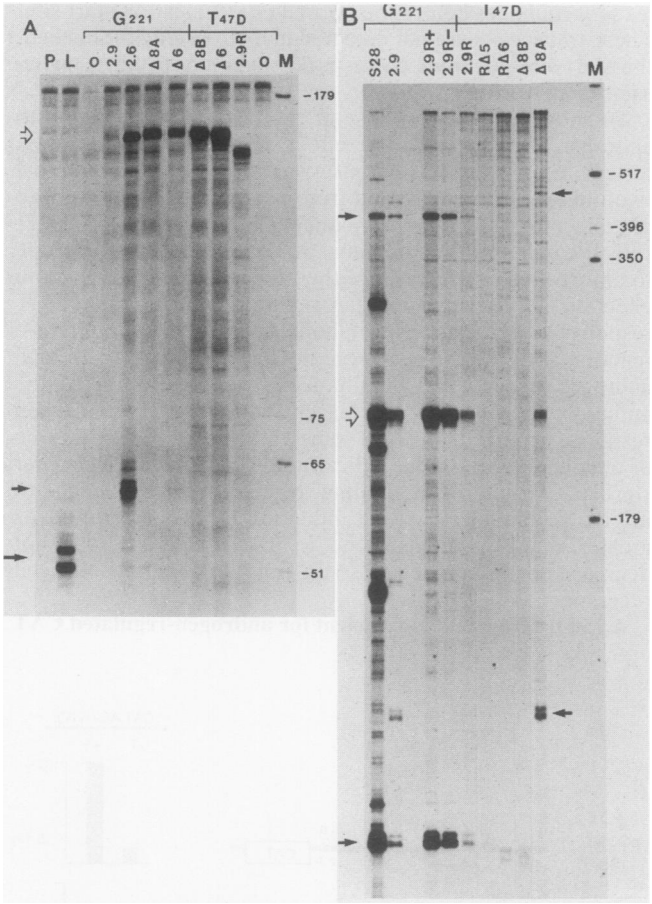

FIG. 5. Transcription start sites in deleted Slp upstream fusion plasmids. RNA from T47D and G221 transfections was mapped for transcripts coming from the correct Slp promoter and from within the *Sma*I-*Xba*I fragment. (A) A 180-nt riboprobe to the Slp cap site was synthesized from a pGem4 plasmid containing Slp 5'-flanking DNA and 50 bp of untranslated region. This probe indicates transcripts that initiate correctly (solid arrows) or start upstream (full protection of probe minus polylinker, open arrow); fusion RNAs protect an extra 12 b of polylinker (short black arrow). G221 analysis is of 50 μg of total RNA from untransfected cells (O) or cells stably transfected with (left to right) pG2.9, pG2.6, pG2.9Δ8A, or pG2.9Δ6. T47D mapping is of 30 μg of total RNA from transient transfections of pG2.9Δ8B and 50 μg from pG2.9Δ6, pG2.9R, or untransfected cells (O). Lane P is probe incubated and digested with no additional RNA; lane L, 5 μg of B10.D2 male liver total RNA; lane M, *Hin*fI-digested pGem3 DNA. (B) The probe in Fig. 3 was used to map transcripts from the *Sma*I-*Xba*I fragment. G221 lanes of total RNA are: 20 μg from a stable line with the intact Slp gene (S28); 20 μg from a stable transformant of pG2.9; 10 μg from a stable line of pG2.9R, grown in the presence (+) or absence (−) of DHT (10 ng/ml) for 48 h. T47D analysis was of cells transiently transfected with the following plasmids: pG2.9R (30 μg of RNA); pG2.9RΔ5 (20 μg); pG2.9RΔ6 (20 μg); pG2.9Δ8B (30 μg); and pG2.9Δ8A (30 μg). Arrows indicate start sites as in Fig. 3, with different positions due to protection of different lengths of polylinker.

expression. Fragments of the 2.9-kb Slp upstream region were fused to the *cat* gene to delineate the responsive region (Fig. 4). The terminal third of the *Bam*HI fragment in the inverted orientation (pG2.9RΔ5) behaved much like the entire fragment: it directed CAT activity that was increased almost fourfold with androgen. The 750-bp *Sma*I-*Xba*I fragment itself directed CAT activity in either orientation (pG2.9RΔ6 and pG2.9Δ9) and showed some hormone response (Table 1). Cryptic internal promoters were apparently stronger in the orientation opposite to that of the natural gene, but activity was obtained in both directions and the induction was similar. Strong induction occurred when the *Sma*I-*Xba*I fragment was directly upstream of the Slp promoter (pG2.9Δ8A); this plasmid reconstructed the natural orientation of promoter and upstream regions, deleting intervening DNA, including the negative element. That response with the *Sma*I-*Xba*I fragment was seen in either orientation and in various positions suggested that an androgen-responsive enhancer resides within it.

TABLE 1. CAT activity and induction in transiently transfected T47D cells

Plasmid	Relative activity[a] (% of pSV2CAT ± SE)	Mean induction[b] ± SEM
pSV2CAT	100	0.96 ± 0.07
pG3CATF	0.20 ± 0.03	1.21 ± 0.09
pG2.9R	15.87 ± 2.61	5.05 ± 0.33
pG2.9RΔ5	1.88 ± 0.57	3.80 ± 0.82
pG2.9RΔ6	9.34 ± 1.28	1.65 ± 0.11
pG2.9Δ9	0.66 ± 0.05	1.56 ± 0.14
pG2.9Δ6	0.29 ± 0.02	1.24 ± 0.09
pG2.9Δ8A	1.35 ± 0.09	3.86 ± 0.19
pG2.9Δ8B	10.04 ± 1.03	1.81 ± 0.25
pG3CRL	1.35 ± 0.56	1.91 ± 0.27
pRΔ6CRL	71.62 ± 21.39	1.61 ± 0.17
pΔ9CRL	9.04 ± 1.65	3.06 ± 0.50

[a] CAT activity in the absence of androgen relative to pSV2CAT (100%) was averaged for four separate transfections in which both the plasmid and pSV2CAT were tested. pSV2CAT in these cells shows about 25% acetylation.
[b] The mean induction was calculated for four of six transfections, with the highest and lowest experimental values discarded.

Variations in basal expression and inducibility of the constructs could be due to use of different promoters. Therefore, transcription start sites were mapped for several constructs to determine whether the *Sma*I-*Xba*I fragment was activating the Slp promoter, which by itself is weak in these cells (pG2.9Δ6). Again, as *cat* mRNA levels are low in transient transfection, stable lines in G221 cells were established for comparison. RNA was mapped with a probe that protects the first 50 bases of both C4 and Slp transcripts (plus 12 bases of fusion mRNAs due to transcribed polylinker sequences in both probe and mRNA). While the authentic cap site was obvious in stable transformants with C4 DNA (pG2.6), it was barely detectable from constructs with extensive Slp DNA (pG2.9) or the Slp promoter alone (pG2.9Δ6) (Fig. 5A). The *Sma*I-*Xba*I fragment did not appear to increase transcription from the Slp promoter (pG2.9Δ8A and pG2.9Δ8B). In fact, for all these plasmids, in stable or transient transfections, most transcripts seemed to start upstream, as the probe (minus polylinker) was fully protected.

As transcripts appeared to initiate upstream even when the Slp promoter was present, start sites within the *Sma*I-*Xba*I fragment were examined. The start sites mapped in pG2.9R divergently oriented to the Slp promoter were also used in the *Sma*I-*Xba*I fragment regardless of its orientation in front of the *cat* gene (Fig. 5B). In fact, they were present in the transfected full gene in clone S28, which makes correctly sized and inducible Slp mRNA. Minor variations in band sizes were due to different lengths of protected polylinker in the various constructs; major start sites still corre-

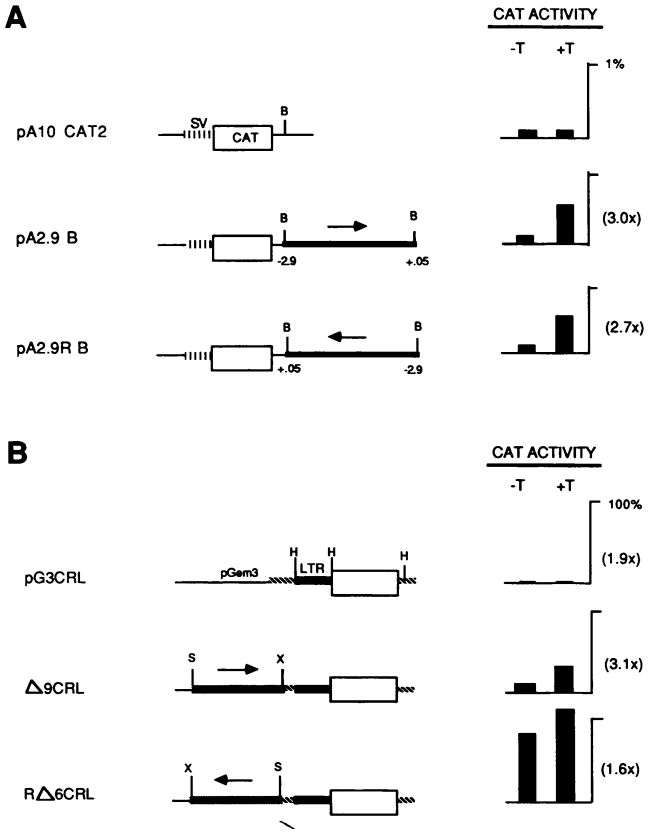

FIG. 6. *cat* fusion constructs with heterologous promoters. The entire Slp upstream *Bam*HI fragment was placed 3′ to the *cat* gene in $pA_{10}CAT2$ (A) and the *Sma*I-*Xba*I fragment was placed 5′ to the RSV promoter in pG3CRL (B) as detailed in the text. CAT activity after transient transfection of T47D cells was analyzed as before. $pA_{10}CAT2$ activity was about 0.2% of pSV2CAT activity. Activity of plasmids in panel B is shown relative to pSV2CAT expression. Induction (fold) is shown in parentheses.

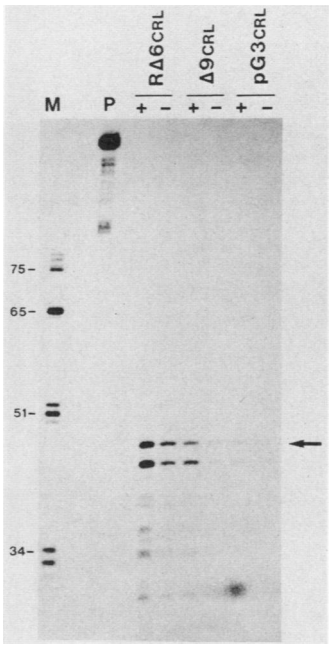

FIG. 7. Enhancement of the RSV promoter by the *Sma*I-*Xba*I fragment. T47D cells were transfected with the indicated plasmids, and RNA was isolated after 48 h with (+) or without (−) DHT. A 90-nt probe was synthesized from a pGem3 plasmid with the RSV LTR, including 40 nt of untranslated region and further 5′ sequences. Thirty micrograms of each total RNA was treated with RNase-free DNase and annealed with probe (P); digestion was with fourfold more RNase in low-salt (100 mM NaCl) buffer. The arrow indicates the pair of prominent RSV start sites.

sponded to sequences residing 2.1 and 2.4 kb from the in vivo Slp cap site. Plasmids with the *Sma*I-*Xba*I fragment in its natural orientation (pG2.9Δ8A and pG2.9Δ9) did not show prominent start sites on the other strand, implying that multiple weak promoters may direct the CAT activity seen with these plasmids. Thus, differences in basal expression of the constructs seem to reflect stronger cryptic promoters in the *Sma*I-*Xba*I fragment in the reverse direction. Hormone induction, however, was seen in both orientations.

The 750-bp fragment can function as an enhancer of heterologous promoters. As the Slp promoter is poorly used, we asked whether the hormone-responsive region could activate heterologous promoters more clearly. When the 2.9-kb *Bam*HI fragment was placed 3′ to the *cat* gene driven by an enhancerless SV40 promoter (in $pA_{10}CAT2$), there was a threefold increase in CAT activity in the presence of androgen (Fig. 6A). Basal-level expression in these plasmids did not increase in the absence of hormone, and thus in these constructs enhancement by Slp sequences was steroid dependent. As the level of expression was so low (although hundreds of counts above the background in the CAT assay), we were unable to determine whether induction was due to increase of transcription from the SV40 promoter. However, it seems unlikely that transcripts initiating at the cryptic promoters within Slp DNA could account for the increase, as such RNAs would have leader regions many kilobases long.

Since this experiment suggested that Slp DNA could enhance a heterologous promoter, we placed the *Sma*I-*Xba*I fragment upstream of a stronger promoter to see whether this would increase effects to levels sufficient for accurate RNA analysis. A partially deleted RSV LTR (16, 18) was placed into pG3CATF, resulting in pG3CRL, and the *Sma*I-*Xba*I fragment was inserted upstream of the LTR in both orientations (Fig. 6B). The fragment increased expression in this vector about 7-fold when it was in the natural orientation and over 50-fold in the opposite orientation. However, the LTR itself was moderately responsive to androgen (1.9-fold); the *Sma*I-*Xba*I fragment in the natural orientation increased induction, whereas in the opposite orientation it did not. Thus, with the strong LTR promoter, enhancement by the Slp fragment was apparent, but hormonal regulation may be complicated by interaction with sequences in the LTR. Interestingly, strong progesterone induction was noted for both plasmids containing the *Sma*I-*Xba*I fragment but not for the LTR alone. The progesterone effect was previously seen with Slp DNA (in pG2.9R) and, unlike the androgen effect, apparently was not affected by the LTR.

When transcription start sites were mapped for the LTR derivatives, enhanced transcription was seen clearly at the correct LTR promoter (Fig. 7). Sites internal to the *Sma*I-*Xba*I fragment may be still somewhat active, but were minor relative to transcripts from the strong LTR

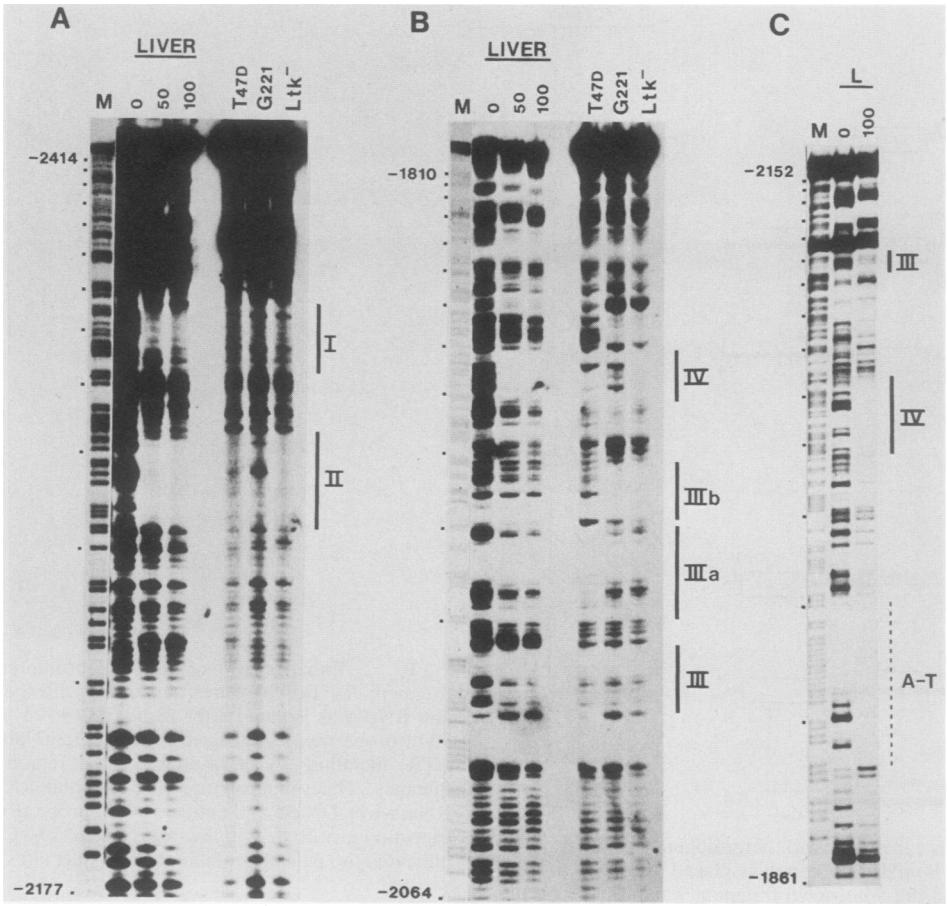

FIG. 8. Binding of nuclear proteins to Slp upstream regions. *Pvu*II-cleaved fragments of the *Sma*I-*Xba*I region were subcloned into plasmid polylinkers for convenience in end-labeling and excision of probe. Probes thus derived were incubated with 0, 50, or 100 μg of liver nuclear extracts or 75 μg of T47D, G221, or Ltk⁻ cell nuclear protein. (A) Fragment B (−2151 to −2414) was ^{32}P-labeled by kinase at the end proximal to the Slp gene by using an *Eco*RI linker site, excised with *Pst*I, and gel purified. Lane M is the A+G sequence reaction of the probe. Protected regions are marked at the right. Dots on the left show 20-base increments. Fragment C (−1809 to −2151) was labeled similarly at its distal (B) or proximal (C) end.

promoter, as fully protected probe was not seen. In this case, then, even in the absence of added hormone, the Slp DNA showed strong enhancement of a heterologous promoter.

Specific protein binding within the *Sma*I-*Xba*I fragment. To substantiate the functional significance of the Slp upstream region to expression in vivo and to localize possible regulatory sequences, we examined the ability of the *Sma*I-*Xba*I fragment to bind nuclear proteins. *Pvu*II cleaves twice within this region, producing fragments, from 5′ to 3′ with respect to Slp transcription, of about 140, 260, and 340 bp (fragments A, B, and C, respectively). In gel shift assays, incubation with liver nuclear extracts caused fragments B and C to migrate more slowly; this retention was competed with an excess of the same fragment but not by nonspecific DNA (data not shown). Fragments B and C did compete with each other. Fragment A did not show significant protein binding.

The sites of protein interaction were characterized by the ability of crude extracts to protect specific sequences from DNase cleavage. Fragments B and C, uniquely labeled at either end, were reacted with nuclear extracts and digested with DNase I. Fragment B showed two distinct windows of protection in the footprint analysis, FPI and FPII (Fig. 8A). FPI spanned about 24 bp centered at −2290 with respect to the Slp promoter and appeared similar whether the extracts were derived from male or female mouse liver or cell lines (T47D, G221, and Ltk⁻). FPII, at about −2230, showed a subtle difference in the sequence protected by liver versus cell extracts; a hypersensitive site in the 20-bp footprint appeared with extract from the two mammary cell lines. The footprints were similar whether the fragment was labeled at the end closest to the Slp gene, as shown, or at the distal end (not shown).

The digestion pattern of fragment C showed two major protected sites, with greater differences seen between the several extracts (Fig. 8B). FPIV, ca. position −1955, was similar in all extracts, with a central hypersensitive site again appearing with the mammary cell line proteins. FPIII showed the greatest variability. With liver extracts, FPIII (seen more clearly when the fragment was labeled at its proximal end [Fig. 8C]) was a small protected region centered at −2040. Additional protection occurred 3′-ward in the human cell line (T47D, FPIIIa) and even further 3′-ward in the mouse cell lines (L and G221, FPIIIb). There was also protection 3′ to FPIV that may be less specific, as this region

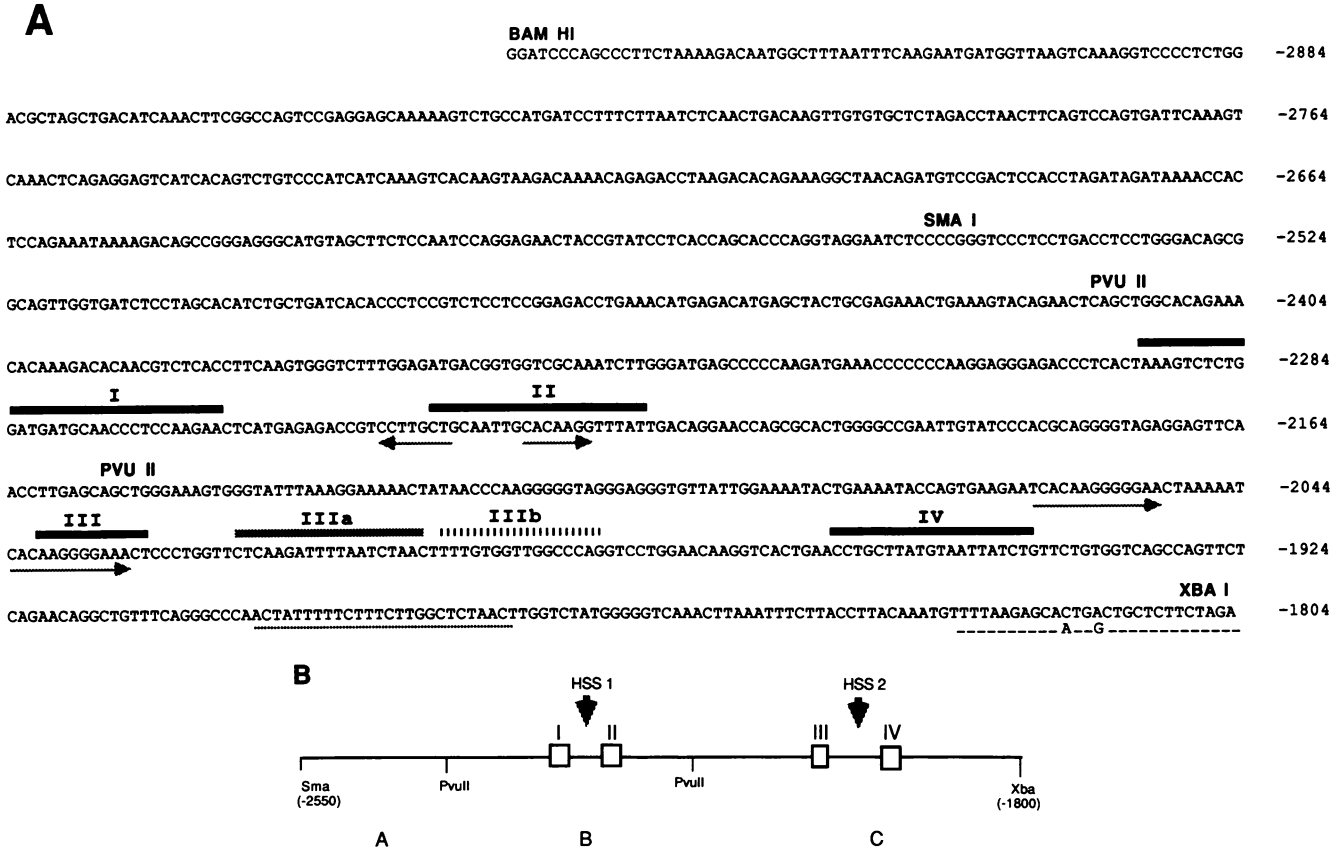

FIG. 9. Sequence of Slp upstream DNA including the enhancer region. (A) Restriction sites used in cloning are shown. Numbers are with respect to the Slp cap site. Solid bars are regions protected by all extracts; FPIIIa was protected by T47D and FPIIIb was protected by G221 and L cell extracts. The dotted line marks an A+T-rich protected sequence. Arrows mark a repeated motif that lay in and close to the footprints. Dashes at the 3′ end show identical bases in C4, with 96% homology extending 3′-ward. (B) Diagram of the *Sma*I-*Xba*I fragment, showing FPI to FPIV and the approximate position of DNase-hypersensitive sites (HSS) (15).

is A+T rich; an apparent footprint shown in Fig. 8C is 70% A+T.

The footprints are shown in the upstream region sequence in Fig. 9. C4 and Slp homology abruptly diverges about 20 bp 5′ to the *Xba*I site (23) of the responsive fragment. Homology to C4 returns several kilobases beyond this breakpoint (5; Stavenhagen, unpublished data). It is interesting that at the end of the homology, 23 (C4) or 25 (Slp) of 26 bp were identical to a sequence found in mouse B2 repetitive sequences as well as in murine leukemia virus (GenBank). Within fragment C, there were some sequences also found in mouse mammary tumor virus (MMTV). Particularly, within FPIV there was a 9-bp sequence that occurred twice about 55 bp upstream of the MMTV cap site, and FPIIIa had an inverted repeat found at −945 in the MMTV LTR, differing only in the 2 central bp. The sequence [CACA AGG(G/T)(G/T)] appeared within FPIII, just upstream of FPIII, and similarly as an inverted repeat in FPII; this sequence is required for liver-specific albumin expression (12, 13). Consensus with known protein-binding sites, such as for Sp1 or NF1, was not seen. As fragments B and C were A+T-rich, some reasonable TATAA boxes occurred in both orientations that may allow the cryptic promoter activity. The significance of these and other sequences requires functional analysis.

DISCUSSION

Slp and C4 are homologous genes that have diverged in the function and regulation of their encoded products. DNA sequences underlying Slp's hormonal regulation should differ in C4, which is not testosterone dependent. In this report, we describe an element residing about 2 kb upstream of the Slp gene that functions as a complex androgen-responsive enhancer.

Androgen-responsive transcription was first evidenced in an inverted Slp promoter fragment that directed *cat* gene expression (35). *cat* mRNA initiated 2.1 and 2.4 kb upstream of the Slp promoter on the opposite strand. These positions coincide with DNase-hypersensitive sites in mouse liver chromatin that are associated with hormonal and developmental regulation of Slp (15). Therefore, we focused on this region as a likely regulatory domain. Gene fusions with deleted Slp upstream regions showed that a 750-bp *Sma*I-*Xba*I fragment was sufficient for hormone-responsive CAT activity in either orientation. Other enhancer properties (activation from a distance and of heterologous promoters) were seen when the entire Slp upstream region was fused 3′ to the *cat* gene or when the *Sma*I-*Xba*I fragment was placed before the RSV LTR. This enhancer is complex, however, in that subregions defined by *Pvu*II cleavage of the *Sma*I-*Xba*I

fragment are inactive (L. Shen and D. M. Robins, unpublished). As there are several protein-binding sites within this region, interaction of distinct elements may be required for effect. Furthermore, interaction of the enhancer with the Slp promoter may be complex and affected by additional regulatory elements.

The Slp upstream fragment showed the greatest enhancing ability when placed before the RSV promoter, where prominent transcripts clearly derived from the RSV start site. In other cases, increase at the RNA level was more difficult to detect, as transient expression was low and *cat* mRNA is unstable (relative to protein). For example, an effect on the Slp promoter when the *Sma*I-*Xba*I fragment was directly upstream was not detectable, as the cryptic promoters mapped within the fragment accounted for most of the transcripts. While this may be a problem of sensitivity, it is also possible that the Slp promoter has some negatively acting sequences associated with it. In addition, it is possible that the strong RSV promoter is able in some way to suppress the internal start sites, making promoter activation more apparent.

When placed upstream of the RSV promoter, the 750-bp *Sma*I-*Xba*I fragment showed strong enhancement (up to 50-fold) in the absence of added hormone. In contrast, when the 2.9-kb Slp *Bam*HI fragment was placed 3′ to the *cat* gene driven by the SV40 promoter, the threefold enhancement was dependent on androgen. Possibly, an effect on basal expression requires proximity to the promoter or is affected by the additional Slp sequences present in the constructs with the SV40 promoter. Inducibility of the LTR-driven constructs was complicated by hormonal response of the viral sequences; induction was increased with the Slp fragment in one orientation but not the other. This contrasts the nature of the Slp enhancer to that of other steroid response elements (39).

One of the more unusual aspects of the *Sma*I-*Xba*I region was the internal cryptic promoters. The major start sites oriented opposite to the Slp promoter were always detected following transfection, regardless of neighboring sequences or whether the DNA was integrated or extrachromosomal. Yet in vivo, transcripts from this region were not found. Instead, this locale is marked by hypersensitive sites that correlate with Slp expression (15). Other cellular enhancers, such as for insulin (9) and immunoglobulin heavy chain (19), also can initiate transcripts. As the Slp upstream region is A+T-rich, RNA polymerase may simply act on promoterlike sequences not protected by protein following transfection. In vivo, these sequences may be unavailable due to the arrangement of specific factors, methylated sites, or differential nucleosome phasing. Such features may be acquired in development but not necessarily in gene transfer. The cryptic promoters may have a more functional role if the action of polymerase keeps the region open until appropriate factors bind. This region's behavior following gene transfer, while dissimilar in some respects to what is seen in vivo, may provide clues to general enhancer function as the distinction between enhancers and promoters becomes increasingly blurred (20).

The Slp upstream enhancer was stronger or more responsive in some contexts than others. To comprehend its role in vivo, other elements of the Slp gene, such as the promoter, must be examined. It is not clear how Slp or C4 promoters are recognized, as TATA boxes and Sp1-binding sites are lacking (7), yet in vivo a single cap site accounts for over 90% of transcripts (29). When intact Slp or C4 genes are transfected, this cap site is used, albeit inefficiently (Kalff and Robins, in preparation). The poorer use of the Slp promoter in the gene fusions may indicate that downstream sequences influence its activity, as recently shown for a papillomavirus gene (37). Alternatively, the promoter may be marked in some way on the chromosome during development.

Another region affecting both Slp and C4 expression is a negative domain at −300 to −450 bp (23). While it may affect the Slp promoter, it does not appear to affect transcription from the upstream region (for instance, pG2.9RΔ5 lacked this domain but was not more active than pG2.9R). This and other elements may have some cell specificity, like the lysozyme gene negative element (36), since flanking DNA (in pG2.6 and pG2.9) allowed CAT expression in L cells (35) and HepG2 cells (23) but not in T47D cells. In G221 stable transformants, expression of pG2.9 was detectable, but it did not respond to hormone. This is perplexing in light of the inducibility of the transfected intact Slp gene in these cells. One possibility is that even further upstream sequences override negative effects to allow induction. However, 11 kb of Slp upstream DNA failed to direct detectable CAT activity in transient assays (M. Kalff, unpublished). Lack of expression or induction may be due to lack of relevant sequences or appropriate cell factors. In addition, multiple elements and regulatory mechanisms may affect Slp expression and may function independently. For instance, the upstream enhancer is revealed in *cat* constructs, whereas the full gene may be influenced additionally by sequences downstream of the promoter, which may act pre- or posttranscriptionally.

Slp regulation in vivo is likely to be complex, and hormones in addition to androgen may be involved in the 100-fold difference in male and female expression (compared to 5-fold induction achieved in transfection). Androgen cannot elicit Slp expression in hypophysectomized mice (6), implying action of pituitary factors. Growth hormone is responsible for sexually dimorphic gene expression in liver (25) but not kidney, where Slp is also expressed in a male-specific fashion at one-tenth the level of liver (15; Cox and Robins, manuscript submitted). Since the transfected gene responds to hormone in mammary cells, sequences sufficient for response are associated with the gene. Further analysis will determine the cell specificity of the enhancer, as well as other sequences affecting Slp expression, and may reveal factors that increase expression and induction.

Analysis of the hormonal response of the enhancer is limited, as few established cell lines contain androgen receptor and none of these are from liver. There is as yet no evidence that the receptor interacts directly with sequences of the enhancer or, for that matter, with other sequences linked to the Slp gene. Some genes known to be transcriptionally activated by steroids do not bind the appropriate receptors and are regulated indirectly (1). The Slp upstream enhancer bound several proteins from diverse nuclear extracts. Three sequences were bound regardless of the extract's source, whereas a fourth region was variable (FPIII). The latter site, protected by liver proteins, was very similar to a sequence required for liver-specific albumin gene expression (12, 13). Regions downstream of this site were protected by mammary and fibroblast cell extracts. The footprints occurred as two pairs at the approximate positions of the DNase-hypersensitive sites. DNA fragments containing either pair of footprints alone, in either orientation in front of the *cat* gene, showed no promoter activity and no enhancement of the RSV promoter (L. Shen and D. M. Robins, unpublished). Thus, unless the *Pvu*II site used to

separate the footprints is within an androgen receptor-binding site, such a sequence may not be present within the tested fragments and the androgen regulation of this enhancer may be indirect. Furthermore, interaction of different elements within the enhancer may be necessary for its activity.

The enhancer we have described upstream of the Slp gene behaves similarly to other complex cellular enhancers (31). Multiple sequence motifs, each capable of binding cellular factors, constitute domains that may interact within the enhancer. While single domains may be inactive, in some cases their multiplication restores activity (18). Furthermore, this activity may differ in specificity from the entire enhancer (32), indicating that combinations of different sequences may lead to varied patterns of regulation. Future work on the Slp enhancer will elucidate how androgen modulates its behavior and how the enhancer and other DNA elements regulate Slp expression in vivo.

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