MOLECULAR AND CELLULAR BIOLOGY, Apr. 1986, p. 993–1001
0270-7306/86/040993-09$02.00/0

Vol. 6, No. 4

Two Different Factors Act Separately or Together To Specify Functionally Distinct Activities at a Single Transcriptional Enhancer

DONALD DEFRANCO† AND KEITH R. YAMAMOTO*

Department of Biochemistry and Biophysics, University of California at San Francisco, San Francisco, California 94143-0448

Received 23 September 1985/Accepted 20 December 1985

The expression of genes fused downstream of the Moloney murine sarcoma virus (MoMSV) long terminal repeat is stimulated by glucocorticoids. We mapped the glucocorticoid response element that conferred this hormonal regulation and found that it is a hormone-dependent transcriptional enhancer, designated S_g; it resides within DNA fragments that also carry a previously described enhancer element (B. Levinson, G. Khoury, G. Vande Woude, and P. Gruss, Nature [London] 295:568–572, 1982), here termed S_a, whose activity is independent of the hormone. Nuclease footprinting revealed that purified glucocorticoid receptor bound at multiple discrete sites within and at the borders of the tandemly repeated sequence motif that defines S_a. The S_a and S_g activities stimulated the apparent efficiency of cognate or heterologous promoter utilization, individually providing modest enhancement and in concert yielding higher levels of activity. A deletion mutant lacking most of the tandem repeat but retaining a single receptor footprint sequence lost S_a activity but still conferred S_g activity. The two enhancer components could also be distinguished physiologically: both were operative within cultured rat fibroblasts, but only S_g activity was detectable in rat exocrine pancreas cells. Therefore, the sequence determinants of S_a and S_g activity may be interdigitated, and when both components are active, the receptor and a putative S_a factor can apparently bind and act simultaneously. We concluded that MoMSV enhancer activity is effected by at least two distinct binding factors, suggesting that combinatorial regulation of promoter function can be mediated even from a single genetic element.

Transcriptional enhancers are DNA sequence elements that can stimulate the efficiency of initiation from linked promoters without strict regard to the position and orientation of the enhancer relative to the transcription start site (11, 39, 49). They are associated with various classes of genes (10, 20, 27) and seem likely to be a common feature of eucaryotic transcription. Two general biological roles can be inferred from present results. Enhancers that are highly active only within particular differentiated cell types may be determinants of tissue-specific gene expression (10, 39, 55). Also, other enhancers mediate specific modulation of transcription in response to certain regulatory molecules (6, 12, 40, 43).

Substantial evidence suggests that diffusible factors may be essential for the activity of all enhancers (5, 35, 44, 47). One interpretation is that enhancers comprise a family of DNA sequences at which cognate transcriptional regulatory proteins specifically bind and act (62, 66). One such enhancer binding factor has been identified and studied in some detail: in the presence of hormonal ligands such as dexamethasone, glucocorticoid receptor protein associates with specific DNA sequences (36, 46) termed glucocorticoid response elements (GREs), resulting in hormone-dependent enhancement at linked promoters (6). Thus, GREs are modulatory enhancers; obviously, other specific protein-DNA interactions could determine cell type-specific promoter activity by a similar strategy.

Enhancer elements seem to encompass rather long (commonly 100 to 200 base pairs [bp]) segments of DNA which often contain multiply repeated sequence motifs (3, 39). For example, specific glucocorticoid receptor binding (37) and GRE activities (6) were first detected and characterized within a 222-bp segment of mouse mammary tumor virus (MTV) DNA; linker scanning mutagenesis of that sequence revealed a consensus octanucleotide that is essential for receptor footprinting and GRE enhancement, and yielded evidence that the receptor may be the only protein whose sequence-specific interaction is required for activity of that enhancer (7, 64; O. Wrange, D. DeFranco, S. Jones, S. Tavtigian, and K. R. Yamamoto, manuscript in preparation). Consistent with this notion is the finding that the element displays no enhancer activity in the absence of bound receptor (6, 67).

In contrast to the apparent one enhancer-one activator arrangement of the MTV GRE, studies with certain other enhancer elements imply that more complex levels of organization and control may also exist. For example, Herbomel et al. (18) showed that one subregion of the polyomavirus enhancer displays relatively strong activity within fibroblasts, whereas a different subregion is particularly active within teratocarcinoma cells, as if the enhancer contains recognition sites for at least two distinct putative *trans*-acting factors. The analysis of such complex enhancers might be facilitated if one were identified in which the glucocorticoid receptor was one of the activating proteins, since GRE enhancer activity can be regulated exogenously with hormone and since the precise DNA sequences involved in receptor binding and action can be directly determined.

Khoury, Gruss, and co-workers (21–23, 48) characterized an enhancer within a tandemly duplicated sequence element upstream from the transcription initiation site within the Moloney murine sarcoma virus long terminal repeat (MoMSV LTR). This activity, here designated S_a, appears to be preferentially operative in murine cells, and a putative activating factor has been inferred from the results of

* Corresponding author.
† Present address: Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260.

transfections (47). Interestingly, Lowy and Scolnick (24) reported several years ago that glucocorticoids stimulate the accumulation of MoMSV RNA in infected cells, consistent with the possibility that MoMSV might harbor a GRE enhancer in addition to its S_a activity. In this report, we show that the MoMSV LTR indeed contains a GRE, and we test its characteristics and examine its spatial and functional relationship to the S_a enhancer.

MATERIALS AND METHODS

Recombinant plasmids. All procedures were performed essentially according to the methods of Maniatis et al. (26). The arrangement of relevant regulatory and structural sequences are described generically by using a four-letter code: the letters 1 and 4 denote enhancers located upstream and downstream, respectively, of a promoter and structural sequence, which are identified by letters 2 and 3, respectively. T refers to a sequence element from herpes simplex thymidine kinase (tk), S refers to a sequence element from MoMSV, V refers to a sequence element from the simian virus 40 (SV40) early region, C is the chloramphenicol acetyltransferase (CAT) coding sequence, and O denotes the absence of a promoter or enhancer element at a given position.

The following plasmids were used for transfection or as starting material for further construction: pCAT3M (21), which is organized as OOCO, i.e., it contains promoterless CAT coding sequences; pSV1CAT (13), which is in the OVCO arrangement, i.e., it contains the SV40 early promoter upstream of CAT coding sequences; pMSVCAT (22), which is an SVCO derivative of pSV1CAT containing the MoMSV enhancer region (see Fig. 2, fragment *iv*) inserted at the *Sph*I site proximal to the SV40 early promoter; pMSV42CAT (48), which is a derivative of pMSVCAT lacking MoMSV sequences upstream of −224 bp from the MoMSV transcription start site (57; see Fig. 2, fragment *v*) and is designated dl-224 SVCO; wtMSV.tk (15), which is in the SSTO configuration, containing the 3′ LTR of MoMSV (see Fig. 2, fragment *i*) fragment fused to the tk coding sequence at +55 bp relative to the normal tk transcription start site; Δ5′-111 MSV.tk (15), which is an OSTO derivative of wtMSV.tk which lacks MoMSV sequences upstream of −111 bp from the MoMSV start site (see Fig. 2, fragment *ii*) and lacks MoMSV enhancer activity; pRSVβgal (M. Walker, University of California, San Francisco), which contains the Rous sarcoma virus (RSV) LTR upstream of the β-galactosidase structural gene (2); pRSVCAT (22), which contains the RSV LTR upstream of the CAT gene; and ptkψwt (29), which contains the tk promoter and a pseudo-wild-type gene with a net 10-bp deletion in the 5′ nontranslated leader.

To construct OTCO, a 164-bp *Bam*HI/*Bgl*II DNA fragment containing tk sequences from −109 to +55 bp (28) was inserted into the *Bgl*II site of pCAT3M, fusing the tk promoter to CAT. The termini of a 346-bp *Xho*II/*Xba*I DNA fragment extending from 147 bp upstream of the MoMSV transcription start site through the remaining LTR sequences and 47 bp of flanking mink cell DNA (8, 21; see Fig. 2, fragment *iii*) were filled and *Bam*HI linked; insertion into the *Bam*HI site of OTCO generated plasmids OTCS.1 and OTCS.2, which carry the MoMSV fragment in the forward and reverse orientation, respectively, 950 bp downstream of CAT coding sequences. The same MoMSV fragment was inserted in both orientations 109 bp upstream of the herpes simplex virus tk gene at a *Bam*HI linker in ptk/Δ5′-109 (28); after partial *Bam*HI and complete *Bgl*II digestion, the resultant 519-bp DNA fragment was inserted into the *Bgl*II site of pCAT3M to yield plasmids STCO.1 and STCO.2.

Cell culture and transfection. Rat XC tk$^-$ and AR4-2J (55; N. W. Jessup and R. J. Hay, In Vitro [Rockville] **16**:212a, 1980) cells were propagated in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Sterile Systems, Inc.). For DNA transfections, cultures at $\cong 5 \times 10^5$ cells per 100-mm dish were provided with fresh medium 4 h before DNA addition; a mixture of a test plasmid and a half- or equimolar amount of an internal control plasmid was prepared as a calcium phosphate precipitate (59) and applied to the cells for 4 h in the presence of 100 μM chloroquine (25), followed by a glycerol shock (34). After DNA removal, cultures were incubated at 37°C for 16 to 48 h in fresh medium with or without 0.1 μM dexamethasone; no systematic effects of hormone treatment were observed on expression from control plasmids or from test plasmids lacking a GRE.

Enzyme assays. CAT enzymatic activity was assayed in clarified cell extracts as previously described (13). The activity of β-galactosidase expressed from cotransfected pRSVβgal was determined in these extracts (2) as an internal control of transfection efficiency. CAT activities in separate transfections were compared by normalizing to the β-galactosidase activities. Under the conditions chosen, expression from the CAT plasmids was unaffected by the presence of cotransfected pRSVβgal.

Primer extension. Total RNA was isolated from transfected cultures as previously described (6), and primer extensions were performed by the method of McKnight et al. (28) by using 5′-terminal-labeled synthetic single-stranded deoxyribonucleotide primers and reverse transcriptase. In transfections with test plasmids SSTO or OSTO, ptkψwt (29) was cotransfected as an internal control, and primer extension was carried out from a synthetic 24-mer corresponding to tk sequences from +55 to +78 relative to the tk transcription initiation site. In transfections with OTCO or OTCS plasmids, pRSVCAT was the internal control, and primer extensions were initiated from a synthetic 24-mer corresponding to CAT sequences from −31 to −8 relative to the CAT translation start site (1).

DNA sequencing and footprinting. Sequence analysis of the MoMSV LTR used in our studies revealed two additional G:C base pairs not included in the originally published sequence (8); the G residues resided in the top strand between −276 and −275 bp, and between −203 and −202 bp relative to the transcription start site. As a result of these changes, the tandemly repeated sequence motifs were 74 and 73 bp, rather than 73 and 72 bp as previously described (23).

Purified glucocorticoid receptor (60, 61) was a generous gift from J. A. Gustafsson, O. Wrange, J. Carlstedt-Duke, and their colleagues (Karolinska Institute, Stockholm). Procedures for DNase I footprinting of in vitro glucocorticoid receptor binding sites on end-labeled DNA fragments were the same as previously described (36).

RESULTS

Nomenclature and experimental strategy. Promoter activities, enhancer activities, or both were assessed in transiently transfected cells either by mapping and quantitation of transcription initiation events by using a primer extension assay or by estimating promoter function from levels of CAT enzyme activity (13) encoded by sequences fused downstream of the promoter. As detailed in the Materials and Methods section, results with both types of assays were

normalized to expression from cotransfected internal control plasmids.

For clarity, the arrangement of relevant regulatory and structural sequences in each recombinant plasmid are described generically by using a four-letter code: letters 1 and 4 denote enhancers placed upstream and downstream, respectively, of a promoter and structural sequence, which are identified by letters 2 and 3, respectively. In this code, T refers to a sequence element from herpes simplex virus tk, S refers to a sequence element from MoMSV, V refers to a sequence element from the SV40 early region, C is the CAT coding sequence, and O denotes the absence of a promoter or enhancer element. For example, SSTO describes plasmids containing the MoMSV enhancer and promoter fused upstream of the tk coding sequence, whereas OTCS describes those in which a tk promoter and CAT structural gene fusion resides upstream of the MoMSV enhancer. Full descriptions of the plasmids are given in the Materials and Methods section (see also Fig. 2).

MoMSV LTR contains a GRE. Levinson et al. (23) localized the MoMSV enhancer within a tandem duplication located between 182 and 330 bp upstream from the transcription initiation site (57) within a 590-bp MoMSV LTR (see the Materials and Methods section). In our initial experiments, we compared MoMSV promoter activity in plasmids of the general arrangement SSTO and OSTO, the former containing an intact MoMSV LTR and the latter lacking LTR sequences upstream of −111 bp, each fused to the tk coding sequences. Each plasmid was cotransfected with an internal control vector carrying a tk promoter and pseudo-wild-type gene (29) into the rat fibroblast cell line XC. Transcripts were quantitated by extension of a synthetic primer containing tk sequences. Labeled fragments of 59 nucleotides, or 69 and 65 nucleotides, corresponding to initiation events at the MoMSV and tk promoters, respectively, were distinguished and quantitated on polyacrylamide gels (Fig. 1). Normalizing to the tkψwt internal control in each transfection, we found that SSTO specified a 25-fold-higher level of initiation than OSTO (Table 1; Fig. 1, lanes b and d), a result consistent with previous reports (22). In transfected cultures treated with 0.1 μM dexamethasone, transcription was increased an additional fourfold with SSTO (Table 1; Fig. 1, compare lanes b and c, and a 24-fold-shorter exposure, lanes g and h, for untreated and hormone-treated cultures, respectively). Hormone had no effect on promoter function in OSTO (Fig. 1, lanes d and e). Thus, a GRE resides within a segment of the MoMSV LTR that also contains the previously defined S_a enhancer element.

MoMSV GRE is an enhancer. To determine whether the MoMSV GRE is an enhancer, a 346-bp *Xho*II/*Xba*I DNA fragment from the MoMSV LTR (Fig. 2, fragment *iii*) that retains complete S_a activity (21, 48) was used to construct STCO and OTCS plasmids containing the inserted fragment upstream (STCO) or downstream (OTCS) of the tk promoter in either its normal (STCO.1, OTCS.1) or inverted (STCO.2, OTCS.2) orientation (see the Materials and Methods section). In each case, S_a enhancer activity was detected in transfected XC cells in measurements of normalized CAT activity (Table 1 and the Materials and Methods section) relative to parallel transfections with OTCO. Table 1 presents data averaged from three separate experiments; as expected, the absolute magnitude of S_a activity differed somewhat as a function of its precise position and orientation in the different constructions, ranging from 4- to 27-fold stimulation in tk promoter activity. Nevertheless, it is clear that dexamethasone treatment of the transfected cultures

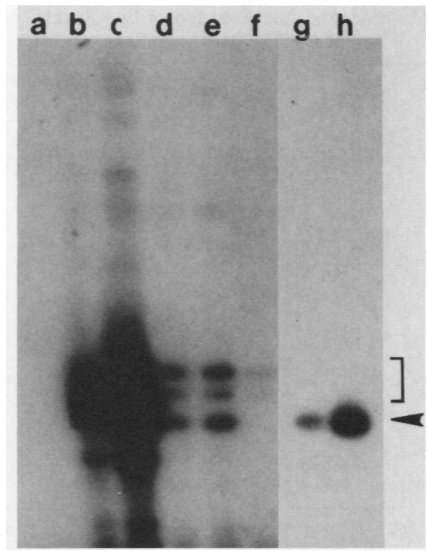

FIG. 1. Identification of a GRE in the MoMSV LTR: primer extension 5′ terminus mapping of transcripts synthesized in transfected XC cells. Rat XC cells were transfected with 5 μg of ptkψwt (lane f) or cotransfected with 5 μg each of ptkψwt and wtMSV.tk (SSTO) (lanes b, c, g, and h), or Δ5′-111 MSV.tk (OSTO) (lanes d and e). Cultures were propagated for 48 h in the absence (lanes b, d, f, and g) or presence (lanes c, e, and h) of 0.1 μM dexamethasone; total RNA was isolated, and 75-μg aliquots were hybridized with an end-labeled (26) synthetic oligonucleotide corresponding to tk sequences from +55 to +78 relative to the tk transcription start site (see the Materials and Methods section). After primer extension with reverse transcriptase, labeled products were separated on a 6% denaturing polyacrylamide gel followed by autoradiography for 48 h (lanes a through f) or 2 h (lanes g and h). Lane a shows the results of a primer extension reaction carried out in the absence of added RNA. Bracket encloses 69- and 65-nucleotide extension products from the ptkψwt internal control plasmid; the arrow marks the 59-nucleotide extension product from the SSTO and OSTO test plasmids.

produced an additional three- to fivefold increase in CAT activity with each of the plasmids. Thus, the MoMSV GRE is indeed an enhancer, here denoted S_g, which coresides with S_a within a 346-bp segment of the LTR.

S_a and S_g can simultaneously enhance a single promoter. Primer extension assays were carried out to determine whether S_a and S_g enhanced initiation from the tk promoter at the same sites or at distinct positions. Therefore, OTCS plasmids were transiently introduced into XC cells (together with pRSVCAT [22], an internal control plasmid which uses the RSV enhancer and promoter to express CAT sequences) in the presence or absence of dexamethasone; the extent and sites of initiation at the respective promoters were monitored by extension of a synthetic primer homologous to a segment of the CAT coding sequences. Transcripts initiated at the RSV promoter in the pRSVCAT plasmid gave rise to a 66-nucleotide extension product, whereas 86- and 82-nucleotide species were produced from the tk promoter transcripts specified by the OTCO and the OTCS plasmids. Figure 3 shows that under conditions in which the OTCO extension products were barely detectable (Fig. 3, lanes a and b), they were readily observed in transfections with either OTCS.1 or OTCS.2 (Fig. 3, lanes c and e) and were an additional four- to fivefold more abundant in cultures treated with dexamethasone (Fig. 3, lanes d and f). Importantly, extension products from OTCS transcripts in control (Fig. 3,

TABLE 1. Mapping and identification of two distinct components of the MoMSV enhancer

Cell line[a]	Plasmid	Relative transcriptional activity[b]	
		S_a	$S_a + S_g$
XC[c]	SSTO	25	100
XC[d]	STCO.1	20	100
	STCO.2	4	20
	OTCS.1	27	92
	OTCS.2	19	61
XC[e]	SVCO	5	40
	dl-224 SVCO	0.8	14
AR4-2J[f]	STCO.1	1	65
	STCO.2	1	8
	OTCS.1	1	16
	OTCS.2	1	39

[a] Cells were cotransfected with test plasmid and internal control plasmid DNAs as described in the Materials and Methods section (also see Fig. 2); data represent average values from three to four separate transfections.

[b] Activity (enhanced) relative to a basal (unenhanced) level of 1; S_a and $S_a + S_g$ denote enhancer activities in cells transfected in the absence and presence of 0.1 μM dexamethasone, respectively. Dexamethasone had no effect on promoter activities in constructions lacking the enhancer. One unit of activity = 1 nmol of chloramphenicol acetylated per min per 50 μg of extract protein at 30°C.

[c] Relative promoter activities were quantitated by primer extension assays and subsequent densitometric scans of autoradiograms.

[d] Relative transcription inferred from assays of CAT activity. Absolute basal CAT activity was 1.3 mU (see footnote *b*).

[e] Relative transcription inferred from assays of CAT activity. Absolute basal CAT activity was 35 mU.

[f] Relative transcription inferred from assays of CAT activity. Absolute basal CAT activity was 0.03 mU.

lanes c and e) and hormone-treated (Fig. 3, lanes d and f) cells were identical. Thus, the precise sites of transcription initiation were not altered by the activity of either S_a or S_g; similar results were obtained in cells transfected with STCO.1 and STCO.2 plasmids (data not shown). Taken together with the results shown in Fig. 1, we conclude that the S_a and S_g enhancers can operate simultaneously upon their cognate MoMSV promoter or upon a heterologous promoter and that they do so by increasing the efficiency of promoter utilization without affecting the positions at which initiation occurs.

MoMSV GRE contains multiple glucocorticoid receptor footprints. It seemed probable that the glucocorticoid-dependent S_g enhancer activity, as with the MTV GREs, is mediated by sequence-specific binding of the receptor. If so, nuclease footprinting experiments with purified receptor could provide a preliminary view of the spatial relationship of the S_a and S_g elements within the MoMSV LTR. Figure 4 shows that multiple DNase I footprints on MoMSV LTR DNA appeared as increasing levels of receptor were added to the reactions. Three discrete regions (footprints 1, 2, and 3) were strongly protected on both strands within an LTR segment 174 to 378 bp upstream of the MoMSV transcription start site. All three first appeared at the same input receptor concentration (Fig. 4A, lane c, and Fig. 4B, lane c), suggesting that receptor bound at each position with roughly equivalent affinity. In addition, somewhat weaker receptor interactions could be discerned between the predominant footprints, particularly on the noncoding (top) strand between footprints 1 and 2, and on the coding (bottom) strand between footprints 2 and 3. Examination of the underlying DNA sequences revealed that each footprint included at least one sequence motif closely related to the consensus octanucleotide $AGA^A_TCAG^A_T$, that has been found within all glucocorticoid receptor footprints (19, 36, 45, 46), and shown to be functional in GRE enhancement (7, 64; Wrange et al., in preparation); the best matches to the consensus sequence reside within the three strong footprints.

As diagrammed in Fig. 2, footprints 1 and 3 bracketed the tandemly repeated sequence element that encompasses the S_a enhancer, and footprint 2 covered the junction of the repeat. This suggests that the MoMSV enhancer is a complex element that can interact simultaneously with at least two different activating factors, and that the sequences that mediate the S_a and S_g activities are interdigitated.

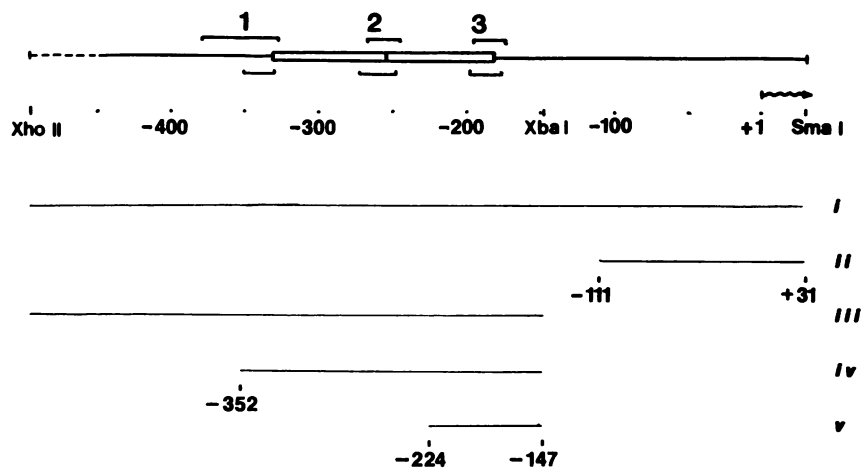

FIG. 2. Structure of the MoMSV LTR. Upper diagram shows a 526-bp *Xho*II/*Sma*I fragment encompassing most of the MoMSV LTR, plus 47 bp of 5′ flanking mink DNA (----), from an integrated provirus isolated and sequenced by Dhar et al. (8). ▭, A 74- to 73-bp tandem repeat associated with the S_a enhancer component (21, 23, 48). Brackets show the positions of the three predominant receptor footprints on the top and bottom strands (see the legend to Fig. 4); transcription initiates at +1 and proceeds rightward. Lines below the map depict the MoMSV LTR fragments used in the present study: *i*, in SSTO; *ii*, in OSTO; *iii*, in STCO.1, STCO.2, OTCS.1, and OTCS.2; *iv*, in SVCO; and *v* in dl-224 SVCO (see the Materials and Methods section).

S_a and S_g enhancer components are functionally distinct and independent. The apparent overlap of the S_a and S_g sequences obviously complicates attempts to uncouple the two activities simply by altering the sequence within that region of the LTR. However, Wrange et al. (in preparation) showed in a study of an MTV GRE containing five receptor footprints that activity is detectable even in subfragments containing only a single footprint sequence. This implied that fragments lacking sequences upstream of footprint 3 in the MoMSV enhancer region might retain S_g activity in the absence of S_a enhancer function. We tested this idea by using SVCO plasmids containing intact or partially deleted MoMSV enhancers fused upstream of an SV40 promoter-CAT gene fusion (48; provided by F. Schulze and P. Gruss, Heidelberg; see the Materials and Methods section). Figure 5A displays CAT assays of rat XC cell extracts from cultures transfected with pMSV42 CAT (48; here designated dl-224 SVCO) which includes only the promoter proximal 42 bp of one tandem repeat plus additional sequences to −147 bp relative to the MoMSV start site (see Fig. 2). In transfected mouse L cells, ≥80% of wild-type S_a activity is lost as a result of this deletion (48), and S_a was completely inactive when dl-224 SVCO was introduced into rat XC cells (Fig. 5A, compare lanes b and f; Table 1); this apparent difference may reflect the preferential activity of S_a in mouse cells (22, 52). In contrast, S_a activity was readily observed in an SVCO plasmid containing an intact MoMSV enhancer fragment. Most importantly, S_g enhancement was observed in both the wild-type and the deletion mutant SVCO constructs. Therefore, the MoMSV LTR sequences that remain in dl-224 SVCO, which include receptor footprint 3, are sufficient to specify hormone-dependent S_g enhancement but fail to elicit S_a activity in transfected rat XC cells. The two activities can therefore be uncoupled genetically, and consistent with previous findings (64; Wrange et al., in preparation), it appears that a single receptor footprint sequence can mediate S_g activity.

As an independent approach, we tested whether the S_a and S_g activities might be distinguished with respect to their relative activities in different cell types. Therefore, the four STCO and OTCS plasmids containing the intact MoMSV

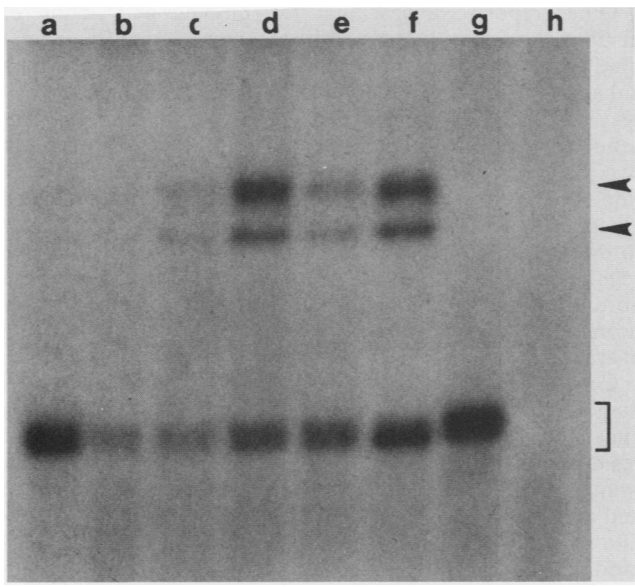

FIG. 3. S_a and S_g can simultaneously enhance a single promoter. XC cells were transfected with 2.5 μg of pRSVCAT (lane g) or cotransfected with 2.5 μg pRSVCAT and 10 μg of OTCO (lanes a and b), OTCS.1. (lanes c and d) or OTCS.2 (lanes e and f). Cultures were incubated for 48 h after transfection in the absence (lanes a, c, e, and g) or presence (lanes b, d, and f) of 0.1 μM dexamethasone. Total RNA was then isolated and 50-μg aliquots were hybridized with an end-labeled synthetic oligonucleotide corresponding to CAT sequences from −31 to −8 relative to the CAT translation start site. After primer extension with reverse transcriptase, labeled products were separated on a 6% denaturing polyacrylamide gel followed by autoradiography for 72 h. Lane h shows the result of a primer extension reaction carried out in the absence of added RNA; bracket demarcates the 66-nucleotide extension product from the pRSVCAT internal control; arrows identify the 86- and 82-nucleotide species generated from the tk promoter transcripts.

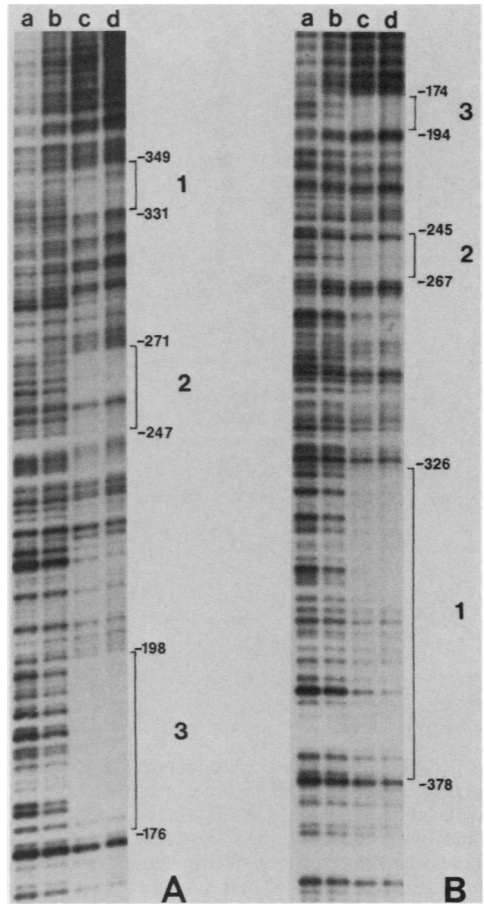

FIG. 4. DNase I footprints of glucocorticoid receptor bound to MoMSV LTR sequences. (A) Coding (bottom) strand. A 390-bp *Hin*dIII/*Eco*RI fragment (5 ng) was isolated from pUC18MSV (constructed by K. Zaret), containing MoMSV LTR sequences upstream of −124, and end labeled at the *Hin*dIII site. The fragment was incubated with 0, 100, 200, and 300 ng (lanes a through d, respectively) of purified glucocorticoid receptor (60, 61) and subjected to partial DNase I digestion as previously described (36). Labeled fragments were electrophoresed in a 6% denaturing polyacrylamide gel together with the products of Maxam-Gilbert sequencing reactions (data not shown). (B) Noncoding (top) strand. A 670-bp *Eco*RI/*Nru*I fragment from STCO.2 was end labeled at the *Eco*RI site and subjected to DNase I footprinting as described above. Three major footprints are bracketed on each strand, and their positions relative to the MoMSV transcription initiation site are shown (Fig. 2).

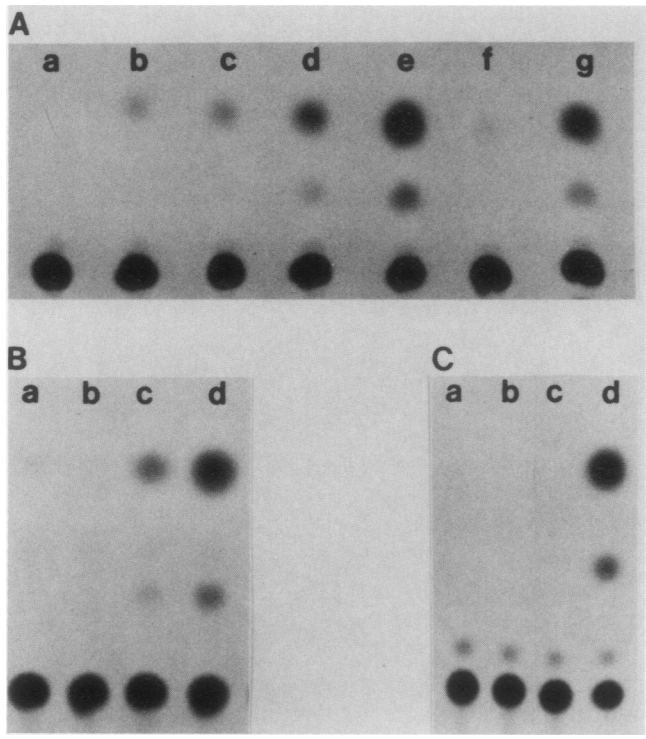

FIG. 5. S_a and S_g enhancer components are functionally distinct and independent. (A) XC cells were cotransfected with 1 μg of pRSVβgal plus 0.5 μg of either CAT3M (lane a), pSV1CAT (OVCO) (lanes b and c), pMSVCAT (SVCO) (lanes d and e), or dl-224 (SVCO) (lanes f and g). After transfection, cultures were propagated for 18 h in the absence (lanes a, b, d, and f) or presence (lanes c, e, and g) of 0.1 μM dexamethasone. CAT activity was assayed in extracts as previously described (13), and chromatograms were autoradiographed for 24 h; signals at the bottom represent unreacted substrate near the origin of the thin-layer chromatograph, and upper spots represent mono- and multiacetylated chloramphenicol products. (B) Transfections were carried out and processed as for panel A, except that 2 μg of OTCO (lanes a and b) or 2 μg of STCO.1 (lanes c and d) was used as the test plasmid in control (lanes a and c) and hormone-treated (lanes b and d) cells. (C) Transfections were carried out and processed as for panel B, except that 10 μg of the test plasmid was used, and AR4-2J was the recipient cell line.

enhancer region were transfected into various rat cell lines. Whereas S_a and S_g both enhanced tk promoter function in XC cells (Fig. 5B and Table 1), only S_g activity was observed in AR4-2J (55; Jessup and Hay, In Vitro [Rockville], 1980), a rat exocrine pancreas cell line (Fig. 5C and Table 1). We concluded that the S_a and S_g components of the MoMSV enhancer can be distinguished and observed independently under appropriate physiological conditions: S_g alone appears to act upon the tk promoter in hormone-treated AR4-2J cells, and S_a activity alone is observed in untreated XC cultures.

DISCUSSION

The known physiological roles of glucocorticoid receptors, coupled with the demonstration that GREs are receptor-dependent enhancers (6, 40, 62), led to the suggestion that the general function of all enhancers might be to serve as specific binding sites for a class of transcriptional regulatory proteins (62, 63). Studies of receptor action at GREs are facilitated by the ability to modulate receptor activity with exogenous hormone, by the selection and characterization of receptor mutations (4, 16, 51, 65), by the purification of receptor to near-homogeneity (60, 61), and by isolation of receptor-specific monoclonal antibodies (9, 32, 58) and cDNA clones (30, 31, 56). By using these tools, it has been shown that the pure protein binds in vitro to specific sequences that mediate GRE enhancer function in vivo, and that hormonal activation of GREs is accompanied by a rapid change in chromatin structure at the positions of receptor binding (38, 67).

Genetic and molecular analyses of the MTV enhancer suggest that the glucocorticoid receptor is the only sequence-specific determinant of its activity (7, 64; Wrange et al., in preparation). In contrast, the MoMSV enhancer is a complex element apparently recognized by at least two distinct activator proteins: an unidentified effector of S_a activity (47), and the glucocorticoid receptor acting upon S_g. It seems likely that both factors recognize multiple sites within the MoMSV enhancer, in view of both our results with receptor footprinting and previous studies suggesting that S_a activity is conferred by several subregions (21, 48). Thus, the simplest interpretation of our present results is that specific binding sites for the receptor and for the putative S_a factor are overlapping and interdigitated and that they can function either separately or concurrently; by this interpretation, it appears that both factors can simultaneously occupy at least a subset of their multiple binding sites at a single MoMSV enhancer element to form a functional complex.

Other reports suggest that the binding and action of distinct regulatory factors within a single complex element may prove to be a general arrangement. Thus, the polyoma enhancer appears to be composed of subdomains that display distinct cell-type tropism (18), an upstream activator sequence in *Saccharomyces cerevisiae* has been divided into two sequence components whose activities require the action of different *trans*-acting gene products (17), and purified glucocorticoid and progesterone receptors bind selectively to overlapping sequences that appear to be involved in the regulation of chicken lysozyme gene transcription by these hormones (42, 54). Indeed, such tight packing of regulatory protein binding sequences is reminiscent of the organization of transcriptional regulatory regions in procaryotes (14, 41).

Consistent with previous work (7, 64), the present results demonstrate clearly that the apparent strength of a given enhancer is a complex function of the cell type in which it resides, the specific promoter to which it is linked, the DNA sequences between the enhancer and promoter, the presence and activity of other enhancers, and the physiological conditions. Although our observations alter an earlier interpretation (7) of transfections of AR4-2J with plasmids containing the MTV and MoMSV enhancers together with the tk promoter, the overall conclusions of that study and the resultant general proposals (63, 64) are supported and extended by our present findings. We speculate that each enhancer element, in association with its specific regulator proteins, might potentiate the activity of a particular transcription factor, and that different enhancers are specific for different factors (63, 64). Given that the nucleotide sequence of each promoter determines which factors limit its intrinsic efficiency, an enhancer, according to this scheme, could relieve rate-limiting steps in the function of some promoters but not those of others. Moreover, this interpretation suggests that multiple enhancers positioned near a given promoter could readily explain combinatorial effects by which relatively few regulatory components can specify many

different cell-specific patterns of expression and control (63, 64).

Since S_a clearly can relieve a rate-limiting step in tk promoter function in XC cells, the simplest interpretation of its failure to act in AR4-2J is that the putative S_a factor may be absent or inactive in the latter cell line; the lack of S_a activity in fusions to several other promoters in AR4-2J (D. Defranco, unpublished data; M. Walker and A. Boulet, personal communication) is consistent with this view. In contrast, both cell lines contained functional glucocorticoid receptors, and in the presence of an active S_a component in XC cells, S_g appeared to stimulate all three promoters to an approximately similar (four- to sevenfold) extent. In AR4-2J, hormonal activation of S_g strongly stimulated tk promoter function in the absence of detectable S_a activity.

These studies furnish evidence that combinatorial regulation can be effected from even a single genetic element. This raises provocative questions about the molecular structure and composition of the various protein-DNA complexes that define and distinguish the activities of such an enhancer and whether protein-protein interactions between the heterologous activators might define functionally unique structures. The cloning (30, 31, 56) and expression of glucocorticoid receptor coding sequences should facilitate approaches to these issues and may also contribute to the isolation and characterization of the putative S_a factor.

The potential to uncouple and genetically dissect the sequences involved in specifying S_a and S_g activities was demonstrated with a large deletion of MoMSV sequences within the enhancer region; it should be possible to extend this analysis to the nucleotide level. In this context, it seems particularly interesting that S_g activity was not detected in the enhancer region of the Moloney murine leukemia virus (33). A comparison of our MoMSV LTR sequence data (8; see the Materials and Methods section) with a published sequence of the Moloney murine leukemia virus enhancer region (53) suggests that the two enhancers may differ in only six positions. That is, the Moloney murine leukemia virus enhancer can be viewed as a mutant of the MoMSV element that has selectively lost S_g activity.

By pursuing these types of molecular and genetic approaches, together with cell-free biochemical studies of transcriptional enhancement (44, 50), it should eventually be possible to dissect the molecular mechanisms by which enhancement operates, and also to discern the biological rationale for this regulatory strategy.

ACKNOWLEDGMENTS

We thank Lou Laimins, Ken Zaret, Barbara Graves, Cori Gorman, Mike Walker, and Frank Schulze for various plasmid DNAs used in this study; Mike Walker, Anne Boulet, and Bob Tjian for oligonucleotide primers; and Orjan Wrange, Jan Carlstedt-Duke, and Jan-Ake Gustafsson for purified glucocorticoid receptor. We are also indebted to Steve McKnight and Peter Gruss for sharing their results before publication and to Roger Miesfeld, Sandro Rusconi, and Jeff Vanderbilt for suggestions regarding the manuscript. Finally, we thank Kathleen Rañeses for expert preparation of the text and Bonnie Maler for help with the figures.

This work was supported by Public Health Service grant CA20535 from the National Institutes of Health; D.D was National Institutes of Health postdoctoral fellow, and K.R.Y. is a recipient of a Dreyfus Teacher/Scholar Award.

LITERATURE CITED

1. **Alton, N., and D. Vapnek.** 1979. Nucleotide sequence analysis of the chloramphenicol resistance transposon Tn*9*. Nature (London) **282**:864–869.
2. **An, G., K. Hidaka, and L. Siminovitch.** 1982. Expression of bacterial β-galactosidase in animal cells. Mol. Cell. Biol. **2**:1628–1632.
3. **Boshart, M., F. Weber, G. Jahn, K. Dorsch-Hasler, B. Fleckenstein, and W. Schaffner.** 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell **41**:521–530.
4. **Bourgeois, S., and J. C. Gasson.** 1985. Genetic and epigenetic bases of glucocorticoid resistance in lymphoid cell lines, p. 311–351. *In* G. Litwack (ed.), Biochemical actions of hormones, vol. 12. Academic Press, Inc., New York.
5. **Cereghini, S., J. Piette, M.-H. Kryszke, and M. Yaniv.** 1985. Interaction of cellular factors with the SV40 and polyoma enhancers, p. 78–85. *In* Y. Gluzman (ed.), Genetic elements regulating initiation of transcription in eukaryotes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. **Chandler, V. L., B. A. Maler, and K. R. Yamamoto.** 1983. DNA sequences bound specifically by glucocorticoid receptor in vivo render a heterologous promoter hormone responsive in vivo. Cell **33**:489–499.
7. **DeFranco, D., O. Wrange, J. Merryweather, and K. R. Yamamoto.** 1985. Biological activity of a glucocorticoid regulated enhancer: DNA sequence requirements and interactions with other transcriptional enhancers. UCLA Symp. Mol. Cell. Biol. **20**:305–321.
8. **Dhar, R., W. L. McClements, L. W. Enquist, and G. F. Vande Woude.** 1980. Nucleotide sequences of integrated Moloney Sarcoma provirus long terminal repeats and their host and viral junctions. Proc. Natl. Acad. Sci. USA **77**:3937–3941.
9. **Gametchu, B., and R. W. Harrison.** 1984. Characterization of a monoclonal antibody to the rat liver glucocorticoid receptor. Endocrinology **114**:274–279.
10. **Gillies, S. D., S. L. Morrison, V. T. Oi, and S. Tonegawa.** 1983. A tissue-specific transcription enhancer element is located in major intron of a rearranged immunoglobulin heavy chain gene. Cell **33**:717–728.
11. **Gluzman, Y., and T. Shenk (ed.).** 1983. Enhancers and eukaryotic gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Goodbourn, S., K. Zinn, and T. Maniatis.** 1985. Human β-interferon gene expression is regulated by an inducible enhancer element. Cell **41**:509–520.
13. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
14. **Gottesman, S.** 1984. Bacterial regulation: global regulatory networks. Annu. Rev. Genet. **18**:415–441.
15. **Graves, B. J., R. N. Eisenman, and S. L. McKnight.** 1985. Delineation of transcriptional control signals within the Moloney murine sarcoma virus long terminal repeat. Mol. Cell. Biol. **5**:1948–1958.
16. **Grove, J. R., and G. M. Ringold.** 1981. Selection of rat hepatoma cells defective in hormone-regulated production of mouse mammary tumor virus RNA. Proc. Natl. Acad. Sci. USA **78**: 4349–4353.
17. **Guarente, L., B. Lalonde, P. Gifford, and E. Alani.** 1984. Distinctly regulated tandem upstream activation sites mediate catabolite repression of the*CYC1* gene of *S. cerevisiae*. Cell **36**:503–511.
18. **Herbomel, P., B. Bourachot, and M. Yaniv.** 1984. Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell **39**:653–662.
19. **Karin, M., A. Haslinger, A. Holtgreve, R. I. Richards, P. Krauter, H. M. Westphal, and M. Beato.** 1984. Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-II_A gene. Nature (London) **308**:513–519.
20. **Labhart, P., and R. H. Reeder.** 1984. Enhancer-like properties of the 60/81 bp elements in the ribosomal gene spacer of *Xenopus laevis*. Cell **37**:285–289.
21. **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.
22. **Laimins, L. A., G. Khoury, C. Gorman, B. Howard, and P. Gruss.** 1982. Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA 79:6453–6457.
23. **Levinson, B., G. Khoury, G. Vande Woude, and P. Gruss.** 1982. Activation of SV40 genome by 72-base pair tandem repeats of Moloney sarcoma virus. Nature (London) 295:568–572.
24. **Lowy, D. R., and E. M. Scolnick.** 1978. Glucocorticoids induce focus formation and increase sarcoma viral expression in a mink cell line that contains a murine sarcoma viral genome. J. Virol. 25:157–163.
25. **Luthman, H., and G. Magnusson.** 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. Nucleic Acids Res. 11:1295–1308.
26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Mattaj, I. W., S. Lienhard, J. Jiricny, and E. M. De Robertis.** 1985. An enhancer-like sequence within the Xenopus U2 gene promoter facilitates the formation of stable transcription complexes. Nature (London) 316:163–167.
28. **McKnight, S. L., E. R. Gavis, R. Kingsbury, and R. Axel.** 1981. Analysis of transcriptional regulatory signals of the HSV thymidine kinase gene: identification of an upstream control region. Cell 25:385–398.
29. **McKnight, S. L., and R. Kingsbury.** 1982. Transcriptional control signals of a eukaryotic protein-coding gene. Science 217:316–324.
30. **Miesfeld, R., S. Okret, A.-C. Wikstrom, O. Wrange, J.-A. Gustafsson, and K. R. Yamamoto.** 1984. Characterization of a steroid hormone receptor gene and mRNA in wild-type and mutant cells. Nature (London) 312:779–781.
31. **Miesfeld, R., S. Rusconi, S. Okret, A.-C. Wikstrom, J.-A. Gustafsson, and K. R. Yamamoto.** 1985. Preliminary analyses of the glucocorticoid receptor gene and its expression: a DNA-binding protein essential for hormone-dependent transcriptional enhancement. UCLA Symp. Mol. Cell. Biol. 30:535–545.
32. **Okret, S., A.-C. Wikstrom, O. Wrange, B. Andersson, and J.-A. Gustafsson.** 1984. Monoclonal antibodies against the rat liver glucocorticoid receptor. Proc. Natl. Acad. Sci. USA 81: 1609–1613.
33. **Overhauser, J., and H. Fan.** 1985. Generation of glucocorticoid-responsive Moloney murine leukemia virus by insertion of regulatory sequences from murine mammary tumor virus into the long terminal repeat. J. Virol. 54:133–144.
34. **Parker, B. A., and G. R. Stark.** 1979. Regulation of simian virus 40 transcription: sensitive analysis of the RNA species present early in infection by virus or viral DNA. J. Virol. 31:360–369.
35. **Parslow, T. G., and D. K. Granner.** 1983. Structure of a nuclease-sensitive region inside the immunoglobulin kappa gene: evidence for a role in gene regulation. Nucleic Acid Res. 11:4775–4792.
36. **Payvar, F., D. DeFranco, G. L. Firestone, B. Edgar, O. Wrange, S. Okret, J.-A. Gustafsson, and K. R. Yamamoto.** 1983. Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region. Cell 35:381–392.
37. **Payvar, F., O. Wrange, J. Carlstedt-Duke, S. Okret, J.-A. Gustafsson, and K. R. Yamamoto.** 1981. Purified glucocorticoid receptors bind selectively in vitro to a cloned DNA fragment whose transcription is regulated by glucocorticoids in vivo. Proc. Natl. Acad. Sci. USA 78:6628–6632.
38. **Peterson, D. O.** 1985. Alterations in chromatin structure associated with glucocorticoid-induced expression of endogenous mouse mammary tumor virus genes. Mol. Cell. Biol. 5: 1104–1110.
39. **Picard, D.** 1985. Viral and cellular transcription enhancers, p. 24–48. *In* N. Maclean (ed.), Oxford surveys on eukaryotic genes, vol. 2. Oxford University Press, Oxford.
40. **Ponta, H., N. Kennedy, P. Skroch, N. E. Hynes, and B. Groner.** 1985. Hormonal response region in the mouse mammary tumor virus long terminal repeat can be dissociated from the proviral promoter and has enhancer properties. Proc. Natl. Acad. Sci. USA 82:1020–1024.
41. **Raibaud, O., and M. Schwartz.** 1984. Positive control of transcription initiation in bacteria. Annu. Rev. Genet. 18:173–206.
42. **Renkawitz, R., G. Shutz, D. von der Ahe, and M. Beato.** 1984. Sequences in the promoter region of the chicken lysozyme gene required for steroid regulation and receptor binding. Cell 37:503–510.
43. **Ryals, J., P. Dierks, H. Ragg, and C. Weissmann.** 1985. A 46-nucleotide promoter segment from an IFN-α gene renders an unrelated promoter inducible by virus. Cell 41:497–507.
44. **Sassone-Corsi, P., A. Wildeman, and P. Chambon.** 1985. A trans-acting factor is responsible for the simian virus 40 enhancer activity in vitro. Nature (London) 313:458–463.
45. **Scheidereit, C., and M. Beato.** 1984. Contacts between hormone receptor and DNA double helix within a glucocorticoid regulatory element of mouse mammary tumor virus. Proc. Natl. Acad. Sci. USA 81:3029–3033.
46. **Scheidereit, C., S. Geisse, H. M. Westphal, and M. Beato.** 1983. The glucocorticoid receptor binds to defined nucleotide sequences near the promoter of mouse mammary tumour virus. Nature (London) 304:749–752.
47. **Scholer, H. R., and P. Gruss.** 1984. Specific interaction between enhancer-containing molecules and cellular components. Cell 36:403–411.
48. **Schulze, F., E. Boehnlein, and P. Gruss.** 1985. Mutational analyses of the Moloney murine sarcoma virus enhancer. DNA 4:193–202.
49. **Serfling, E., M. Jasin, and W. Schaffner.** 1985. Enhancers and eukaryotic gene transcription. Trends Genet. 1:224–230.
50. **Sergeant, A., D. Bohmann, H. Zentgraf, H. Weiher, and W. Keller.** 1984. A transcription enhancer acts in vitro over distances of hundreds of base-pairs on both circular and linear templates but not on chromatin-reconstituted DNA. J. Mol. Biol. 180:577–600.
51. **Sibley, C. H., and G. M. Tomkins.** 1974. Isolation of lymphoma cell variants resistant to killing by glucocorticoids. Cell 2:221–227.
52. **Spandidos, D. A., and N. M. Wilkie.** 1983. Host-specificities of papilloma-virus, Moloney murine sarcoma virus and simian virus 40 enhancer sequences. EMBO J. 2:1193–1199.
53. **Van Beveren, C., E. Rands, S. K. Chattopadhyay, D. R. Lowy, and I. M. Verma.** 1982. Long terminal repeat of murine retroviral DNAs: sequence analysis, host-proviral junctions, and preintegration site. J. Virol. 41:542–556.
54. **von der Ahe, D., S. Janich, C. Scheidereit, R. Renkawitz, G. Schutz, and M. Beato.** 1985. Glucocorticoid and progesterone receptors bind to the same sites in two hormonally regulated promoters. Nature (London) 313:706–709.
55. **Walker, M. D., T. Edlund, A. M. Boulet, and W. J. Rutter.** 1983. Cell-specific expression controlled by the 5′-flanking region of insulin and chymotrypsin genes. Nature (London) 306:557–561.
56. **Weinberger, C., S. M. Hollenberg, E. S. Ong, J. M. Harmon, S. T. Brower, J. Cidlowski, E. B. Thompson, M. G. Rosenfeld, and R. M. Evans.** 1985. Identification of human glucocorticoid receptor complementary DNA clones by epitope selection. Science 228:740–742.
57. **Weiss, R., N. Teich, H. Varmus, and J. Coffin (ed.).** 1982. RNA tumor viruses, p. 1329–1332. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
58. **Westphal, H. M., G. Moldenhauer, and M. Beato.** 1982. Monoclonal antibodies to the rat liver glucocorticoid receptor. EMBO J. 1:1467–1471.
59. **Wigler, M., S. Silverstein, L.-S. Lee, A. Pellicer, Y.-C. Chang, and R. Axel.** 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell 11:223–232.
60. **Wrange, O., J. Carlstedt-Duke, and J.-A. Gustafsson.** 1979. Purification of the glucocorticoid receptor from rat liver cytosol. J. Biol. Chem. 254:9284–9290.
61. **Wrange, O., S. Okret, M. Radojcic, J. Carlstedt-Duke, and J.-A. Gustafsson.** 1984. Characterization of the purified glucocorticoid receptor from rat liver cytosol. J. Biol. Chem. 259:4534–4541.
62. **Yamamoto, K. R.** 1984. Transcriptional enhancement by spe-

cific regulatory protein:DNA complexes, p. 79–92. *In* H. S. Ginsberg and H. J. Vogel (ed.), Transfer and expression of eukaryotic genes. Academic Press, Inc., New York.

63. **Yamamoto, K. R.** 1985. Steroid receptor regulated transcription of specific genes and gene networks. Annu. Rev. Genet. **19**:209–252.
64. **Yamamoto, K. R.** 1985. Hormone-dependent transcriptional enhancement and its implications for mechanisms of multifactor gene regulation, p. 131–148. *In* L. Bogorad and G. Adelman (ed.), Molecular developmental biology: expressing foreign genes. 43rd Symposium of the Society for Developmental Biology. Alan Liss, Inc., New York.
65. **Yamamoto, K. R., U. Gehring, M. R. Stampfer, and C. H. Sibley.** 1976. Genetic approaches to steroid hormone action. Recent Prog. Horm. Res. **32**:3–32.
66. **Yamamoto, K. R., B. A. Maler, and V. L. Chandler.** 1983. Fusions of a glucocorticoid response element to the tk promoter region: hormone regulated enhancement of transcription initiation, p. 165–169. *In* Y. Gluzman and T. Shenk (ed.), Enhancers and eukaryotic gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
67. **Zaret, K. S., and K. R. Yamamoto.** 1984. Reversible and persistent changes in chromatin structure accompany activation of glucocorticoid-dependent enhancer element. Cell **38**:29–38.