

Hormonal response region in the mouse mammary tumor virus long terminal repeat can be dissociated from the proviral promoter and has enhancer properties

(glucocorticoid hormone induction/mouse mammary tumor virus/long terminal repeat/promoter/enhancer)

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ABSTRACT The proviral DNA of mouse mammary tumor virus (MMTV) contains a regulatory region closely associated with its promoter, which subjects transcription to the control of glucocorticoid hormones. Delimitation analysis of a chimeric MMTV long terminal repeat-thymidine kinase gene (LTR-*tk*) has shown that the hormonal regulation sequence is confined to 202 nucleotides preceding the LTR-specific RNA initiation site. A second RNA initiation site (*tk*-specific mRNA) placed close to the regulatory MMTV sequence by *in vitro* recombination is also subjected to hormonal stimulation in transfected cells. A series of plasmids with deletions around the LTR cap site progressing from 3' to 5' was made and functionally tested. *In vitro* deletion of MMTV LTR sequences comprising the RNA initiation sequence and the "TATA" box do not effect hormonal regulation at the *tk*-specific mRNA start site. Nucleotides up to position -59 from the LTR initiation site could be deleted without influence on the glucocorticoid regulation, whereas deletions to position -65 abolished the hormonal effect on the *tk* gene transcription. A short MMTV LTR segment containing nucleotides -236 to -52 from the LTR initiation site was recombined with the *tk* gene or the α -globin gene. This fragment confers hormonal inducibility onto the heterologous genes over distances of 0.4 or 1.1 kilobases. The hormonal response region functions when it is placed either 5' or 3' of the regulated gene in both of the possible orientations and is reminiscent of an enhancer sequence.

Glucocorticoid hormone action has been studied in its molecular details in a variety of systems (1). One of the most fruitful approaches has been the use of the proviral gene of mouse mammary tumor virus (MMTV) (2). The initial observation that a hormonal regulation signal is encoded by the proviral DNA (3, 4) was followed by the localization of the regulatory sequence in the MMTV long terminal repeat (LTR) (5, 6). The delimitation of this sequence from the 5' side by *in vitro* deletion of MMTV LTR DNA showed that 202 nucleotides preceding the LTR-specific RNA initiation site are sufficient to confer maximal induction to the transcription of LTR RNA (7-9) and to a thymidine kinase (TK) gene (*tk*) recombined with the MMTV LTR *in vitro* (7). Additional information on the mechanism of hormone action was obtained by the definition of the level of regulation. The rate of RNA initiation was found to be affected, and the functional glucocorticoid receptor was shown to be required (10). *In vitro* interaction between the glucocorticoid receptor and MMTV LTR DNA revealed preferential binding sites in the sequence found to be important for regulation *in vivo* (11, 12). These *in vivo* and *in vitro* experiments both point out the significance of DNA sequences located about 200 nucleo-

tides 5' of the LTR RNA initiation site but do not define the involvement of the proviral promoter region. The observation that hormonal regulation can be conferred to an adjacent gene and that induction can be measured accurately at the *tk* initiation site (7) made possible the experimental test of this question. A chimeric LTR-*tk* gene containing a LTR as well as a *tk* gene promoter was deleted in the LTR promoter sequences, the deletions progressing in the 3'-to-5' orientation. This deletion series defines the 3' border of the glucocorticoid response region between -59 and -65 of the LTR RNA initiation site.

A combination of a 5' and a 3' deletion mutant yielded a fragment of 184 nucleotides of LTR sequence (-52 to -236) with purely regulatory properties. This sequence (HRE = hormone response element) confers inducibility to a *tk* gene and to a α -globin gene. Therefore, inducibility is not promoter specific and is maintained over a distance of several hundred nucleotides. Chimeric HRE-globin constructions that contain the HRE element either on the 5' or 3' end in both of the possible orientations remain hormonally inducible. The relative position and orientation of the HRE with respect to the regulated gene can be altered without effect on the hormonal response.

MATERIALS AND METHODS

Construction of Chimeric Plasmids. *pPS* deletions. The plasmid *pPS* is based on the plasmid *p2.6* (7). The *Bam*HI fragment in the *env* sequences was removed (figure 1 in ref. 7). We subcloned an *Eco*RI fragment containing the 5' *env*-LTR 3' and flanking mouse sequences into *pBR322*. The resulting plasmid contains a unique *Bst*EII site in the LTR, 27 base pairs (bp) from the 3' end. This plasmid was linearized by *Bst*EII digestion, and deletions were introduced by *BAL*-31 nuclease digestion. Molecules of desired length were isolated by preparative gel electrophoresis. Plasmid DNA was prepared, and the extent of the deletions was determined by sequence analysis. The *Eco*RI fragments with deletions around the *Bst*EII site were used to substitute the original 5' *env*-LTR 3' *Eco*RI fragment in plasmid *pPS*. The resulting deletion series ($\Delta 3'$) contains 5' LTR sequences up to positions +24, +7, -9, and -52 with respect to the RNA initiation site at position +1.

***pNK* deletions.** The *pNK* deletion series is based on *pGR7* (13). This is a LTR deletion plasmid in which sequences 3' of position +40 in the LTR had been eliminated. This deletion had been constructed originally for our sequence analysis (13). This plasmid was linearized at its unique *Hind*III site, which was introduced by linker ligation at position +40. De-

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Abbreviations: LTR, long terminal repeat; MMTV, mouse mammary tumor virus; *tk*, gene for thymidine kinase (TK); HRE, hormone response element; bp, base pair(s); kb, kilobase(s)

letions were introduced by digestion with BAL-31 exonuclease. *EcoRI* linkers were ligated to the endpoints of the deletions, and plasmid DNAs were prepared. An *EcoRI* fragment of plasmid Mtk2 (14) containing the structural *tk* gene was introduced into the *EcoRI* sites of the deletion plasmids. Plasmids were selected in which the transcriptional orientation of the LTR and the *tk* gene was the same. The deletion series ($\Delta 3'$) contains LTR sequences from the 5' site up to positions +3, -47, -59, -65, and -123 with respect to the LTR initiation site.

pHRE-*tk* plasmid. The plasmid pPS $\Delta 3'$ (-52) was combined with the plasmid p2.6 $\Delta 5'$ (-236) (7) to yield plasmid pHRE-*tk* in the following way: the plasmid pPS $\Delta 3'$ (-52) was digested with *Sac* I, and a restriction fragment was isolated that contained 56 bp of LTR sequences, 144 bp of mouse sequences, and 622 bp of *tk* sequences. This fragment was used to substitute the respective fragment in p2.6 $\Delta 5'$ (-236), and a clone was isolated in which the insert had the same orientation than in the original plasmid. This construct was named pHRE-*tk* and contained the following sequences: 445 bp of *env* sequences, 184 bp of LTR sequences contributed by $\Delta 5'$ (-236) and $\Delta 3'$ (-52), 144 bp of mouse sequences, and the *tk* gene.

pHRE-globin constructs. An *EcoRI* fragment containing the mouse α -globin gene (15) was inserted into the *EcoRI* site of pSV-2gpt (17). This plasmid was provided to us by Erwin Wagner (European Molecular Biology Laboratory, Heidelberg). The plasmid contains two *EcoRI* sites on either side of the α -globin gene and was linearized by a limited *EcoRI* digest. The linearized molecules were ligated with the *env*-HRE *EcoRI* fragment isolated from the pHRE-*tk* plasmid. Bacteria were transformed, and various plasmid DNAs were examined for the site of integration of the inserted DNA fragment and for their orientation. Four different plasmids were isolated, each of which contained the *env*-HRE insert in one of the two possible positions and two possible orientations.

Functional Analysis of the Chimeric Plasmids. Transformation of LTK⁻ cells. Plasmids containing the *tk* gene were introduced into LTK⁻ cells by using the calcium phosphate precipitation technique; 50–100 LTK⁺ transformants were selected in hypoxanthine/aminopterin/thymidine medium, pooled, and grown into mass culture. Constructs with the globin gene were cotransfected with pSV2-neo (16); 50–100 LTK transformants resistant against the antibiotic G418 were grown into mass culture. Transfected cells were hormonally induced by growth in 1 μ M dexamethasone for 16 hr before harvesting.

Analysis of the mRNA transcripts. The correctly initiated RNA transcripts were analyzed by nuclease S1 mapping technique. For mapping the *tk* start, we used a single-stranded *tk* fragment 131 nucleotides long, end labeled at the *Bgl* II site 56 nucleotides 3' of the *tk* start (7). Total cellular RNA (50 μ g) was hybridized with 0.03 pmol of this fragment at 41°C in 80% (vol/vol) formamide/0.4 M CaCl₂/40 mM Pipes, pH 6.5/5 mM EDTA. The nuclease S1-resistant DNA was run on a 6.5% polyacrylamide sequencing gel. For mapping the globin start, we used a *Pst* I-*Hae* III fragment 104 bp long that was end-labeled at the *Hae* III site 90 bp downstream from the α -globin cap site. The hybridization conditions were identical to those described for the *tk*-specific mRNA, except that the temperature for hybridization was 45°C.

RESULTS

Chimeric gene constructions between the LTR of MMTV and selectable indicator genes have been shown to be hormonally inducible. A fragment that contains 202 nucleotides 5' of the LTR start site is sufficient for glucocorticoid regula-

tion (7–9). Most chimeric constructions have used the LTR RNA initiation for determining hormonal inducibility (5, 6). One chimeric LTR-*tk* gene tested contains two RNA initiation sites, one in the LTR and a second one representing the authentic *tk*-specific RNA start site (18). Both start sites are used in transfected cells, and the transcription of both resulting RNA molecules is under hormonal control (7, 18). The presence of a hormonally controlled initiation site outside the LTR allows the determination of the sequence requirements from the 3' side—i.e., the question can be asked if the LTR promoter structure with the TATA box and cap site is part of the regulation region.

A Series of 3' Deletions ($\Delta 3'$) in Chimeric MMTV LTR-*tk* Genes. A number of LTR-*tk* plasmids were constructed. Fig. 1A shows two series of deletion mutants. The pPS series is based on plasmid 2.6 (18), which contains the 3' LTR of an exogenously integrated proviral copy from GR tumor cells (*mtv-2* locus; ref. 19). The deleted regions are indicated by the arrows in Fig. 1A. The endpoints of the deletions were determined by DNA sequencing. The numbers refer to the 5' borders of the deletions and indicate the position with respect to the RNA initiation site. $\Delta 3'$ (+24) and $\Delta 3'$ (+7) retain the LTR promoter sequence and the initiation site. $\Delta 3'$ (-9) lacks the RNA orientation site, and $\Delta 3'$ (-52) lacks the TATA sequence. The LTR sequences 5' of the indicated positions are present. The pNK series is based on the 3' LTR of an endogenous provirus of the GR mouse (*mtv-8* locus; ref. 19).

Differently from the pPS series, which contains about 1.5 kilobases (kb) of proviral envelope sequences (*EcoRI*-*Bam*HI fragment; figure 1 in ref. 7), the LTR sequences are bordered by pBR322 sequences in the pNK series. The *Bgl* II site delimiting the proviral DNA to the 5' side is located 38 nucleotides 5' of the LTR-*env* border (20). The pNK deletion series extends further than the pPS series to positions $\Delta 3'$ (-59), -(-65) and -(-123). A detailed description of the construction of these deletion mutants and the origin of the plasmids used is given in *Materials and Methods*.

The Proviral Promoter Region and RNA Initiation Site Are Not Required for Glucocorticoid Regulation. To test the biological capabilities encoded in each of the plasmid constructions shown in Fig. 1A, they were transfected into mouse LTK⁻ cells. These cells contain functional glucocorticoid receptors (21) and have previously been used for the study of glucocorticoid hormone action (3, 22). Mass cultures of stable TK⁺ transformants were isolated. This selection is not dependent upon transcription initiating in the LTR segment of the chimeric genes since each construction contains a functional *tk* promoter. A *tk* gene fragment up to position -77 (*EcoRI* site) is present. The *tk*-specific mRNA derived from the chimeric LTR-*tk* genes initiates at the authentic *tk* start site (7). Since transcription from the *tk* initiation site is hormone inducible in cells transfected with LTR-*tk* chimeras, the presence of a regulatory sequence can be tested independently from LTR-initiated RNA.

Single-strand-specific nuclease mapping experiments (7, 23) were carried out with RNA extracted from transfected cells grown in the absence and presence of dexamethasone. The DNA probe used in these experiments was labeled at the 5' end of the *Bgl* II site located 56 nucleotides downstream of the *tk* mRNA initiation site (7). Fig. 1B shows that all of the deletions derived from the pPS series retain hormonal inducibility. The signal of 56 nucleotides indicating authentically initiated *tk* mRNA is enhanced in all cases in cells grown in the presence of dexamethasone. The first three deletion constructs [pPS, $\Delta 3'$ (+24), $\Delta 3'$ (+7)] contain the MMTV LTR promoter and initiation sequence. The deletion construct $\Delta 3'$ (-9) lacks the initiation site and $\Delta 3'$ (-52) lacks the LTR initiation site and the TATA box. The pNK deletion series extends these results. The plasmids retaining the MMTV

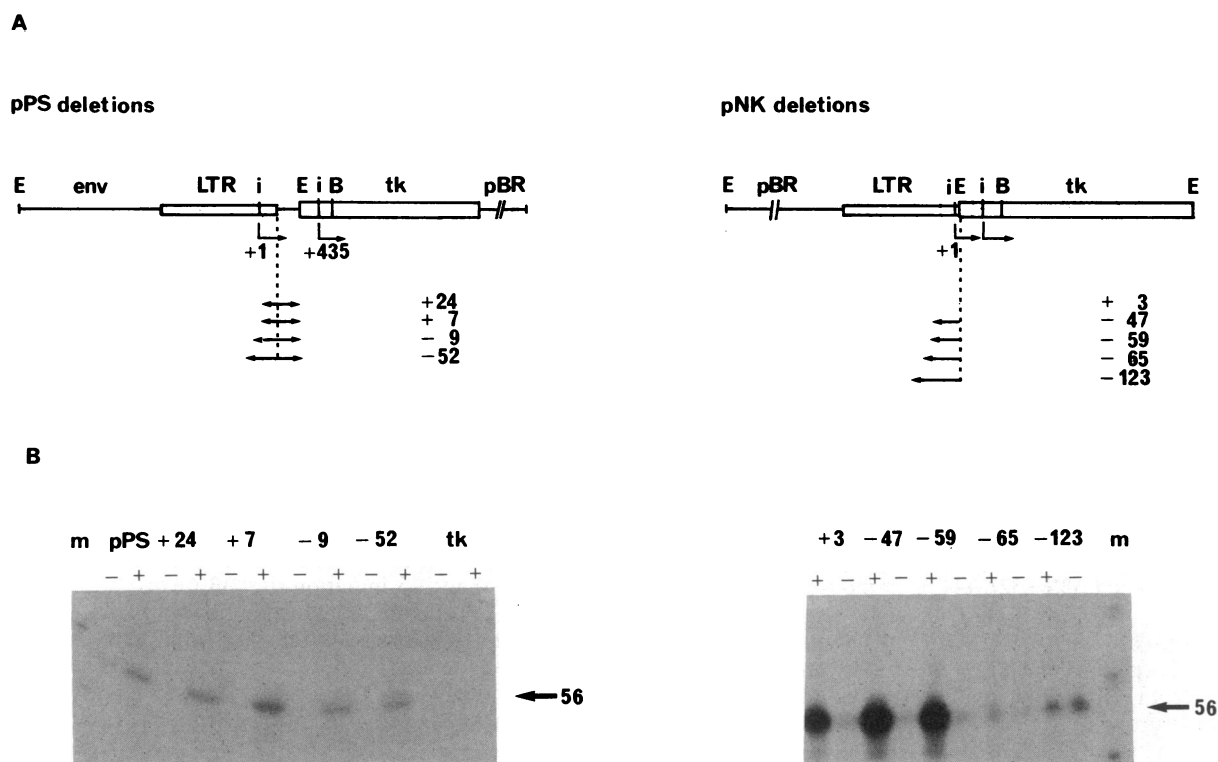


FIG. 1. Deletion mutants around the LTR RNA initiation site in chimeric plasmids of the MMTV LTR and the *tk* gene. (A) Schematic representation of two sets of deletions. The constructions are described in *Materials and Methods*. E, *Eco*RI restriction site; B, *Bgl* II restriction site; i, start of transcription (in the LTR at nucleotide +1). Vertical bars indicate the extent of the deleted DNA fragment in individual plasmids, and numbers indicate the 5' border of the deletions, relative to the transcription initiation in the LTR. (B) Nuclease S1 mapping of the pPS and pNK transcripts initiating at the *tk* start site. Total RNA (50 μ g) from transfected cells (lanes -) or transfected cells treated with 1 μ M dexamethasone (lanes +) were hybridized at 41°C with 3×10^{-2} pmol of 32 P-end-labeled single-stranded *Eco*RI-*Bgl* II *tk* probe. After treatment with nuclease S1, the nuclease-resistant DNA was electrophoresed on a 6.5% polyacrylamide gel. Lanes: m, pBR322 digested with *Hae* III and end-labeled with 32 P; tk, RNA from cells transfected with plasmid DNA of the *tk* gene cloned into pBR322. The numbers above the lanes refer to the deletion plasmids in A. The arrow at 56 nucleotides indicates the size of the expected nuclease S1-resistant fragment.

LTR initiation site [$\Delta 3'(+3)$] as well as the ones in which initiation site and TATA box are deleted [$\Delta 3'(-47)$, and $\Delta 3'(-59)$] are hormone inducible. Deletion plasmids $\Delta 3'(-65)$ and $\Delta 3'(-123)$ lose hormonal responsiveness. Therefore, these deletion mutants define the 3' border of the hormonal regulation element to be located between position -59 and position -65.

A 184-bp LTR Fragment Confers Hormonal Induction. MMTV LTR fragments have been shown to confer inducibility to adjacent promoters. A $\Delta 5'$ series delimited the required sequence to 202 nucleotides 5' of the RNA initiation site (7) and the $\Delta 3'$ series shows that sequences up to position -59 are essential. We combined a -236 deletion from the $\Delta 5'$ series with the -52 deletion from the $\Delta 3'$ series (Fig. 2A). This combination yields a LTR DNA fragment of 184 bp (HRE). To test if these sequences are sufficient to confer hormonal regulation, they were recombined with the *tk* gene and transfected into L cells. Fig. 2B shows that inducibility of mRNA initiated at the authentic *tk* mRNA start site could be detected. The HRE (-236 to -52) is in itself sufficient to exert regulation, and no additional proviral sequences are required. A possible contribution of the 450 nucleotides of *env* sequences present in the element to hormonal induction is ruled out by the results obtained with deletion plasmids of the pNK series, which do not contain *env* sequences.

The Hormonal Response Region Can Act Like a Conditional Enhancer. The effect of position, orientation, and distance of the HRE with respect to the regulated gene and promoter sequence was tested. For this purpose the mouse α -globin gene was recombined with the HRE as shown in Fig. 3. A plasmid containing the α -globin gene flanked by two *Eco*RI sites was used. These *Eco*RI sites are located about 1.1 kb 5'

of the α -globin cap site and about 1.1 kb 3' of the α -globin cap site, respectively. The HRE contained in an *Eco*RI fragment of about 0.7 kb (Fig. 3) was inserted 5' of the α -globin gene in both orientations (Fig. 3, constructs 1 and 2) and 3' of the globin gene in both orientations (Fig. 3, constructs 3 and 4). The α -globin gene-containing plasmid without LTR sequences (Fig. 3, construct 5) served as a control in these experiments. The five constructs shown in Fig. 3 were transfected into L cells, and stable transfectants were isolated. These transfectants were grown in the absence and presence of glucocorticoid hormone, and the transcriptional activity at the α -globin RNA start was quantitated by single-strand-specific nuclease mapping. Accurately initiated α -globin mRNA protects a sequence of 90 nucleotides. All four constructs containing the HRE show hormonal inducibility (Fig. 3, lanes 1-4) of the α -globin gene transcripts. No increase of α -globin mRNA in the presence of hormone is observed in construct 5, which is devoid of MMTV LTR DNA. The effect of the HRE is thus independent of the orientation with respect to the regulated gene, functions at the 5' or 3' end, and can act over a distance of more than 1 kb. However, the presence of hormone is required to exert the enhancing activity on transcription.

DISCUSSION

The identification of regulatory elements that modulate the efficiency of utilization of eukaryotic polymerase II promoter signals has received much recent attention. Suitable assay systems *in vivo* and *in vitro* (24, 25) have allowed the definition of promoter-associated transcription signals. One group of signals that has been studied in considerable detail, re-

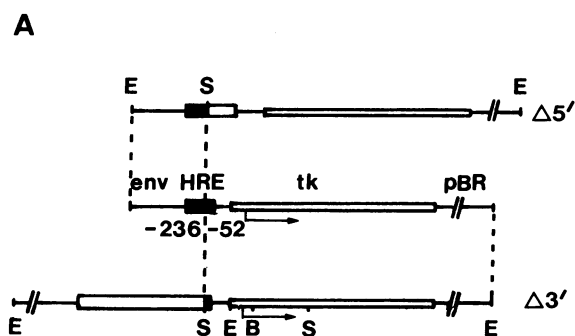
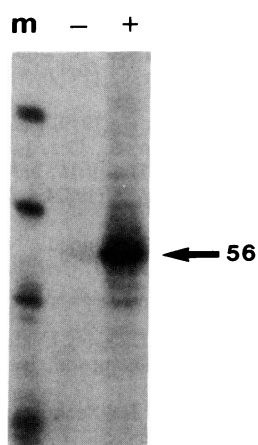

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FIG. 2. (A) Construction of the pHRE-tk plasmid E, *EcoRI* restriction site; B, *Bgl* II restriction site; S, *Sac* I restriction site; $\Delta 5'$ and $\Delta 3'$, 3' and 5' deletions as described in *Materials and Methods*. (B) Nuclease S1 mapping of *tk* transcripts from pHRE-tk DNA. Total RNA (50 μ g) from transfected L cells was probed for correctly initiated *tk* transcripts as described in Fig. 1. Cells were grown in the absence (lane -) or presence (lane +) of 1 μ M dexamethasone. Lane m contains *Hae* III-digested pBR322 32 P-end-labeled marker DNA. The arrow at 56 nucleotides indicates the size of the expected nuclease S1-resistant fragment.

sponds to distinct stimuli. This allows known inducers of gene transcription to be correlated with regulatory DNA sequences. Stimuli as diverse as heat shock (26), heavy metals (27), heme (28), and steroid hormones (2, 29, 30) have been investigated. Sequences that are important for the biological response to steroid hormones have been delimited by *in vitro* recombination and gene transfer procedures (7, 29, 30). These sequences also show a preferential interaction with steroid receptors (30–32). Two strong and two weak binding sites for activated glucocorticoid receptor to a 120-nucleotide region (positions -72 to -192) have been found upstream of the RNA initiation site of the MMTV LTR (11, 12). A hexanucleotide sequence 5' T-G-T-T-C-T 3' is found in all four binding regions (31). The guanosine residues of these hexanucleotides are protected by the receptor protein from methylation by dimethylsulfate (31). This finding, the occurrence of the 5' T-G-T-T-C-T 3' sequence in the region essential for glucocorticoid receptor binding, and regulation of the lysozyme gene (although in an inverse orientation) and a similar sequence in the human metallothionein II_A gene (5' T-G-T-T-C-C 3'; refs. 27 and 30) make it likely that this motif is important for glucocorticoid receptor binding. Proteins

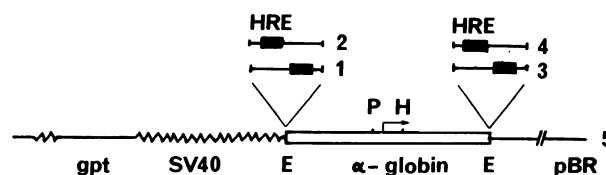
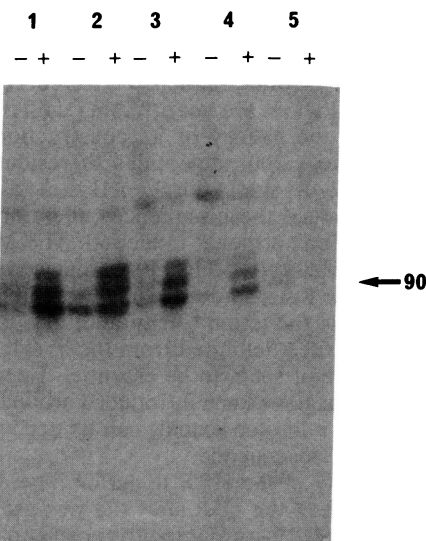
A

B


FIG. 3. Nuclease S1 mapping of α -globin mRNA transcribed from constructs containing the HRE. (Upper) Four different plasmids containing the α -globin gene and the HRE fragment are schematically shown. Their construction is described in *Materials and Methods*. The *EcoRI* fragment containing the HRE was isolated from the pHRE-tk plasmid (Fig. 2). The different positions and orientations of the insertion of this fragment are indicated and numbered 1, 2, 3, and 4. In construct 1 and 2, the HRE is located at the 5' side of the α -globin gene in the *syn* orientation (construct 1) and the *anti* orientation (construct 2). In construct 3 and 4 the HRE is located at the 3' side of the α -globin gene in the *syn* orientation (construct 3) and the *anti* orientation (construct 4). The plasmid without an inserted HRE fragment is construct 5. P, *Pst* I restriction site; H, *Hae* III restriction site. Total RNA (50 μ g) from transfected cells were hybridized with 3×10^{-2} pmol of 32 P-end-labeled DNA probe at 45°C. The probe used was the 104-bp *Hae* III-*Pst* I fragment. After treatment of the hybridization reaction with nuclease S1, the nuclease-resistant DNA was separated on a gel. The cells were grown in the absence (lanes -) or presence (lanes +) of 1 μ M dexamethasone. The numbers above the lanes correspond to the different plasmids used for transfection shown in A. The arrow points to the position of the 90-nucleotides expected as the nuclease S1-resistant fragment for correctly initiated α -globin mRNA. The upper band corresponds to the 104-nucleotide probe.

that are required for the transcription of specific genes and that bind to regions just upstream of the regulated promoter have been described for the *Drosophila* gene for heat shock protein 70 (26) and the simian virus 40 early region (33). In this respect the activated glucocorticoid receptor can be viewed as a gene-specific transcription factor that acts in concert with RNA polymerase II and possibly other general transcription factors.

Glucocorticoid hormone regulation of a 202-nucleotide segment preceding the LTR cap site is not restricted to LTR-initiated RNA but extends to a downstream heterologous promoter (7, 34). This observation has allowed a further delimitation of the minimal regulatory sequence requirement. The construction and analysis of two deletion series of chi-

meric plasmids described here placed the 3' border of the HRE between positions -59 and -65 from the LTR RNA initiation site. If the definition of the promoter region is restricted to the proximal signals for transcription initiation (TATA box and cap site), the hormonal response element can be uncoupled from the promoter. Both deletion series are based on the same principal: ligation of MMTV LTR sequences derived from a region 5' of the proviral promoter to the *tk* gene and quantitation of authentic *tk* transcripts in transfected cells grown in the absence and presence of glucocorticoid hormone. Since the pPS series is based on a MMTV proviral clone derived from the *mtv-2* locus and the pNK series is based on a proviral clone from the *mtv-8* locus and since consistent results are obtained, we conclude that the minor nucleotide sequence differences found in the two MMTV LTRs (13) are not affecting the HRE. Furthermore, because of the history of its construction, the pPS series contains about 400 nucleotides of residual *env* sequences (from the *EcoRI* site to the *BamHI* site) (20). The pNK series does not contain these sequences; therefore, they are inconsequential for hormonal induction. The MMTV RNA start site up to position -59 has been replaced by the equivalent region of the Rous sarcoma virus promoter without effect on the hormone induction (9). In addition, the integrity of the entire sequence delimited from the 5' side (-202) or 3' side (-59) does not seem to be essential. Internal deletions and substitutions have been introduced around position -107 (9, 35) and, to a limited extent, can be accommodated without functional consequence.

The ability of the HRE to act on heterologous promoters independent of the LTR start site was used to test the flexibility of the HRE-promoter arrangement. Distance, orientation, and 5' and 3' location with respect to the regulated promoter could be varied without loss of hormonal response. This property of the HRE is reminiscent of another class of regulatory elements, the enhancers. The discovery of viral enhancers (37), which are little restricted with respect to cell type and species of the responsive cells, was followed by the description of enhancers that are only active in particular cell types. These specialized enhancers are associated with differentiation-specific gene expression in, for instance, B lymphocytes (38) or exocrine and endocrine pancreatic cells (36). The functional similarity of the HRE is apparent with respect to the relative position, distance, and orientation independence. The dependence, however, of the HRE function on glucocorticoid hormone would classify the HRE as a conditional enhancer. Therefore, three types of enhancers can be defined: (i) enhancers that act in a broad spectrum of responsive cells, (ii) enhancers that are dependent on differentiation-specific host factors (39), and (iii) enhancers that are dependent on external stimuli such as steroid hormones. The HRE belongs to the third group and can act on heterologous promoters in cells that contain glucocorticoid receptors and are grown in the presence of hormone. Additional, unknown, superimposed regulatory mechanisms may explain the discrepancy between the restricted expression of MMTV in a few murine tissues (40) and the promiscuous expression and stimulation of MMTV in a large variety of transfected and infected cell types from various species *in vitro*.

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