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Delimitation of a DNA Sequence Which Confers Inducibility by Glucocorticoid Hormones

Bernd Groner, Peter Herrlich, Nick Kennedy, Helmut Ponta, Ursula Rahmsdorf, and Nancy E. Hynes

Kernforschungszentrum Karlsruhe, Institut für Genetik und für Toxikologie von Spaltstoffen, Postfach 3640, D-7500 Karlsruhe 1, Federal Republic of Germany

A chimeric long terminal repeat-thymidine kinase (LTR-tk) gene has been used to define the sequence requirements for glucocorticoid induction of gene expression. The original LTR-tk gene contains an entire mouse mammary tumor virus (MMTV) LTR preceding the tk gene. This gene can be expressed in a hormone-responsive fashion upon transfection into L tk− cells to produce a chimeric LTR-tk mRNA. Stepwise deletion of nucleotide sequences 5′ of the viral RNA initiation site revealed that 202 nucleotides upstream of the viral cap site are sufficient for the hormonal regulation. Deletion of 5′ sequences up to 59 nucleotides upstream of the viral cap site abolished RNA initiation in the LTR and hormonal induction.

Key words: glucocorticoid action, gene transfer, mouse mammary tumor virus, thymidine kinase gene

The expression of mouse mammary tumor virus (MMTV) in cultured cells has been recognized as an important model system for the study of the mechanism of action of glucocorticoid hormones [1,2]. Gene cloning and DNA-mediated proviral gene transfer experiments have shown that a DNA sequence responsible for the hormonal induction of MMTV is present within the proviral gene [3,4]. The combination of the long terminal repeat (LTR) region of MMTV with either the p21 gene of Ha-MuSV [5], the dihydrofolate reductase gene of mouse [6], or the thymidine kinase gene of HSV [7] has shown that the hormonal inducibility can be conferred to at least three other genes located 3′ of the MMTV LTR region. The LTR therefore contains the regulatory information for the hormonal response. The LTR sequence of MMTV comprises 1,328 nucleotides [8,9]. An open reading frame of 960 nucleotides is located at its 5′ side. A viral RNA initiation site has been detected 134 nucleotides from the 3′ end. Because sequences located 5′ of the RNA cap site and the TATA box have been recognized in several other genes to be required for maximal and accurate initiation of transcription [10–12], it is reasonable to suspect regulatory

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signals involved in the hormonal response in this region of the proviral gene. We have investigated the biological function of DNA sequences preceding the RNA initiation site in the LTR by construction of specific deletion mutants of the LTR-tk chimeric gene, reintroduction into cultured cells, and their transcription in the absence and presence of dexamethasone. Deletion of the LTR sequences up to 202 nucleotides 5′ of the RNA initiation site did not affect transcription and hormone inducibility. Deletion of LTR sequences up to 59 nucleotides upstream of the cap site abolishes transcription from the LTR initiation site and its hormonal regulation. These experiments functionally define the borders of the regulatory region which specifies hormonal regulation.

MATERIALS AND METHODS

A MMTV LTR-tk Chimeric Plasmid and Introduction of Specific Deletions

The chimeric LTR-tk gene (shown in Fig. 1A) was constructed as described [7]. A Eco RI fragment of exogenous proviral DNA, cloned in a λ-vector [13: Fig. 4, clone 1] consisting of the env region of MMTV, the right (3′) LTR and about 200 nucleotides of flanking mouse genomic DNA, was joined to the tk gene of HSV [14]. The fragment of HSV DNA cloned in the hybrid plasmid M2 [14] was cut with Pvu II recloned in the Pvu II site of pBR 322 in a clockwise orientation with respect to the direction of transcription of the tk gene [15]. This plasmid is designated tk M 103. 10

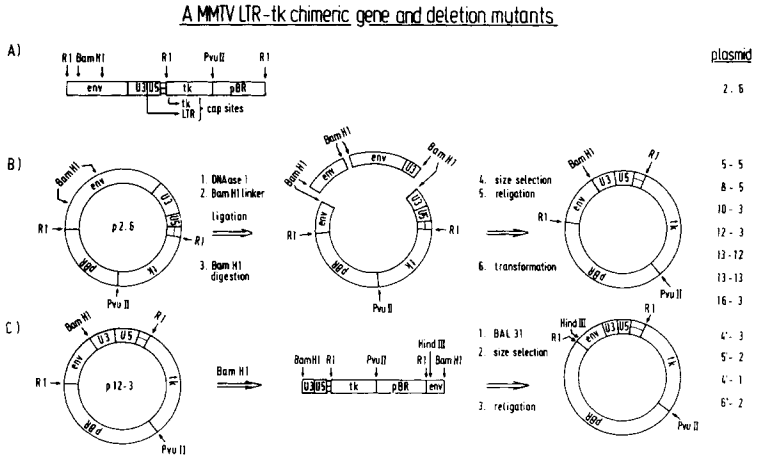

Fig. 1. A) Chimeric MMTV LTR-tk gene. The construction of the chimeric plasmid is described in detail in the text. The MMTV LTR-tk gene is shown for clarity in a linear representation. B) Schematic outline of strategy used for the introduction of targeted deletions by DNAse I and BAM HI linker ligation. The method is detailed in the Results and Methods sections. C) Schematic outline of strategy used for the introduction of targeted deletions by nuclease BAL 31. The method is based on the presence of a single Bam HI site in p 12-3 and nucleotides are removed from the remaining env and U3 regions. Details are described in the Results and Methods sections. Abbreviations: env, envelope glycoprotein region of the MMTV provirus; U3 and U5, long terminal repeat (LTR) region of MMTV; ⊟, mouse DNA flanking the proviral integration site (200 nucleotides); tk, thymidine kinase gene of herpes simplex virus; pBR, partial pBR 322 sequence comprising the origin of replication and the ampicillin-resistance gene.

μg of λ-MMTV clone 1 [13] were digested with Eco RI and electrophoresed on a 0.9% agarose gel (low gelling temperature agarose, Sigma). The 4.2-kilobase (kb) fragment was visualised by UV light after ethidium bromide staining and the fragment was cut out from the gel (gel slice a). Five micrograms of tk M 103 were digested with Eco RI, phenol-extracted, and precipitated with ethanol. The fragments were then treated with bovine intestine alkaline phosphatase (Boehringer) in 50 mM Tris-HCl, pH 8. The enzyme was inactivated by heating at 65°C and the restriction fragments were electrophoresed on a 0.9% agarose gel. The fragments were visualised after ethidium bromide staining and the 4.2-kb fragment containing the tk gene and the pBR 322 sequences from nucleotide 2,067 to 4,360 [16] was removed from the gel (gel slice b). Gel slices a and b were melted at 65°C and 7.5 μl of each were mixed at 42°C. Four microliters of a 5× concentrated ligation buffer (0.18 M Tris-HCl, pH 6.8; 40 mM $MgCl_2$; 50 mM DTT; 2 mM ATP) and 2 units of T4 DNA ligase (Boehringer) were added. After incubation for 12 hr at 15°C the agarose was melted again at 65°C and 25 μl of TCM (0.1 M Tris-HCl, pH 7.5; 0.1 M $CaCl_2$; 0.1 M $MgCl_2$) and 30 μl of TAE (40 mM Tris-acetate, pH 8.1; 20 mM sodium acetate; 2 mM EDTA) were added. Seventy-five microliters of ligated DNA were used to transform 125 μl of Ca^{2+}-treated bacteria (Hb 101), and ampicillin-resistant colonies were selected. These colonies contained two plasmids. One plasmid, p 2.6, contains the MMTV and tk segments in the same orientation with respect to the direction of transcription of both genes. The second plasmid, p 2.5 (not shown), contains the two genes in the opposite transcriptional orientation.

To introduce specific deletions in the LTR region of p 2.6, 50 μg of plasmid DNA were digested with 800 pg of DNAase I in 500 μl of 20 mM Tris-HCl pH 7.6, 1.5 mM $MnCl_2$ at 25°C for 9 min [17]. About 40% of the molecules were linearized. Linear molecules of 8.4 kb (length of plasmid 2.6) were recovered after preparative gel electrophoresis [13]. The DNA was treated with 10 units of T4 DNA polymerase for 30 min at 37°C in 100 μl of 10 mM Tris-HCl, pH 8; 10 mM $MgCl_2$; 10 mM 2-mercaptoethanol; 0.1 mM EDTA; 30 mM ammonium sulfate; 300 μg/ml gelatin; and 0.2 mM deoxynucleotide triphosphates. Unincorporated deoxynucleotide triphosphates were removed on a Sephadex G-50 column and the DNA was further purified by DEAE cellulose chromatography. Synthetic oligonucleotide linkers containing a Bam Hl recognition site (BRL) were phosphorylated with ^{32}P-ATP and T4 polynucleotide kinase. A tenfold molar excess of Bam Hl linkers was ligated to the linearized p 2.6 DNA with T4 DNA ligase. The DNA was digested with Bam Hl and electrophoresed on a low gelling temperature agarose gel (Sigma). About 20 1-mm slices containing DNA molecules from 4.2 to 7.3 kb were separated. Thirty microliters of each gel slice was melted at 65°C and the DNA contained was circularized by ligation at 15°C for 18 hr. The DNA was transfected into bacteria and ampicillin-resistant bacterial clones were selected. Plasmids 5–5, 8–5, 10–3, 12–3, 13–12, 13–13, and 16–3 (Fig. 3) were chosen for further experimentation.

Additional deletion mutants were constructed starting with DNA of p 12–3. Ten grams of p 12–3 DNA were linearized with Bam Hl. The DNA was incubated with 2 units of BAL 31 nuclease in 200 μl at 30°C for various times and the digestion was monitored by gel electrophoresis. About one base pair was removed per second. The deletions of desired length were selected by preparative gel electrophoresis. The DNA was recircularized with T4 DNA ligase and bacteria were transformed. Plasmids 4′–3, 5′–2, 4′–1, and 6′–2 were further investigated.

Transfection of LTR-tk DNA Into L tk– Cells and Quantitation of Transcripts

One hundred nanograms of LTR-tk DNA (plasmids shown in Fig. 3), linearized with Pvu I, were cotransfected with 10 μg of salmon sperm carrier DNA onto L tk– cells [18,19]. Thymidine kinase positive cells were selected in HAT medium [19] and the cell clones were grown into mass culture. RNA and DNA was prepared [3] and the RNA was further fractionated into poly(A)+ and poly(A)–RNA. To 5 μg of yeast carrier RNA 0.1, 0.5, 1 and 2 μg of poly(A)+RNA was added and ethanol-precipitated. The precipitate was redissolved in 20 μl of 50% formamide; 6% formaldehyde; 90 mM Tris-borate, pH 8.3; and 2.5 mM EDTA. The RNA was denatured at 65°C for 3 min and diluted with 180 μl of 10× SSC (1.5 M NaCl, 0.3 M Na acetate). The RNA was dotted onto a nitrocellulose filter (presoaked in 10× SSC) using a hybridot manifold device (BRL, No. 1050 MM). Each dot was washed with 200 μl 10× SSC, the filter was air-dried and baked at 80°C for 2 hr in a vacuum oven. The filters were pretreated as described previously [20] and hybridized to a LTR or a tk-specific radioactive probe. The LTR probe is a 0.6-kb Pst I fragment comprising 400 nucleotides of the 3′ end of the LTR and about 200 nucleotides derived from the gag region of clone GR 40 [3]. The tk probe comprises a 2.0-kb Pvu II fragment which contains the coding region of the tk gene [15]. Both probes were nick-translated to a specific activity of 5×10^7 cpm/μg with ^{32}P dCTP and used for hybridisation as described previously [13]. Following autoradiography, the hybridisation signals were cut from the nitrocellulose filters, eluted in 750 μl 10 mM Tris-HCl, pH 7.5, 0.05% SDS by boiling for 10 min and counted in 10 ml of Insta-Gel (Packard).

RESULTS

A Chimeric LTR-tk Gene and Introduction of Deletions

A chimeric gene was constructed which contains a 4-kb Eco RI fragment of MMTV proviral DNA (env gene and R-LTR) [13], the tk gene of HSV [14] and a segment of pBR 322 (Fig. 1A). Upon introduction of this chimeric gene into mouse L cells, hormone responsive expression of the U5 region of the LTR and the adjacent tk gene could be observed [7]. The LTR is 1,328 nucleotides long and the RNA initiation site is located only 134 nucleotides from its 3′ end [8]. We investigated the possible presence of regulatory signals 5′ of the viral cap site by stepwise deletion of nucleotide sequences from the LTR. The two experimental strategies used are shown in Figure 1B,C. The chimeric gene env-LTR-tk-pBR 322 (p 2.6) is shown in Figure 1B. This plasmid DNA was linearized at random positions with DNAse I and synthetic oligonucleotides containing a Bam Hl recognition site were ligated to ends of the plasmid DNA. The DNA was then digested with Bam Hl. This enzyme cuts the env region of MMTV twice (Fig. 1B). Size selection, recircularisation, and bacterial transformation allowed us to obtain plasmids derived from p 2.6 which have lost defined regions of env and LTR DNA. We isolated seven plasmids (p 5–5, 8–5, 10–3, 12–3, 13–3, 13–12, 16–3) and determined the nucleotide sequence around the single remaining Bam Hl site. Comparison with the known nucleotide sequence of the MMTV LTR [9] revealed the exact position of the initial DNAse I cut. The size of LTR sequences remaining are listed in Figure 3 with respect to the 3′ border of the LTR and the RNA initiation site.

A second strategy for the deletion of nucleotide sequences 5′ to the LTR promoter region is shown in Figure 1C. DNA of p 12.3, retaining 583 nucleotides of LTR sequence (Fig. 3), was linearized with Bam Hl. Digestion of linearized DNA with the exonuclease BAL 31 for various times removed DNA of increasing length from the end of the molecules. The digestion was visualized by gel electrophoresis of the shortened plasmid DNA. Fractions with the desired extent of digestion were recircularized and plasmid DNA was prepared in transformed bacteria. Plasmids 4′–3, 5′–2, 4′–1, and 6′–2 were obtained and the end points of the remaining LTR sequences were determined by DNA sequencing and comparison to the complete LTR sequence.

Introduction of LTR-tk Genes Into L-Cells and Quantitation of LTR and tk Transcripts

The chimeric plasmid LTR-tk (p 2.6) and the deletion mutants in Figure 1B,C were transfected into mouse L tk− cells. HAT selection was applied and stable tk+ transfectants were selected. This selection is possible without the functional assistance of the promoter located within the MMTV LTR. The tk gene is fused to the mouse DNA at an Eco RI site located 77 nucleotides upstream from the authentic tk RNA cap site [12]. Although the transfection efficiency of a tk gene deleted to the −77 position is impeded [12], the tk promoter still functions properly [Hynes et al, in press]. The presence of the LTR-tk gene in an uninterrupted fashion in the selected L-cell clones was demonstrated by digestion of the genomic DNA with restriction enzymes, Southern blotting, and hybridisation to a tk-specific probe. DNA of cell clones transfected with plasmids 5–5, 8–5, 10–3, 12–3, 13–13, 13–12, and 16–3 were digested with Bam Hl and Pvu II and the expected fragments of 3.5, 3.3, 2.9, 2.8, 2.6, 2.3, and 2.2 kb could be detected. DNA from cells transfected with plasmids 4′–3, 5′–2, 4′–1, and 6′–2 were digested with Hind III and Pvu II, and fragments of 2.5, 2.3, 2.1, and 2.1 kb were observed with a tk probe (not shown).

The RNA of the cell clones transfected with the LTR-tk genes was analyzed to test the functional capabilities encoded by the deletion mutants. A dot-hybridisation procedure was used to quantitate and compare the amount of specific RNA sequences present in the transfected cell clones grown in the absence and presence of dexamethasone. Poly(A)+RNA was isolated from the cells and increasing amounts were dotted onto a nitrocellulose filter (Fig. 2). The filter-bound RNA was hybridized to a nick-translated LTR probe and a tk probe and the signals obtained were visualized by autoradiography. The hybridized LTR and tk DNA was then eluted from the filter and the radioactivity was counted. The results obtained with RNA from the p 2.6 transfected cells is shown in Figure 2. A linear relationship between the amount of RNA applied and the hybridisation signal can be observed. Comparison of the slopes of the curves obtained with RNA from cells grown in the absence and presence of dexamethasone was used as a measure of hormonal induction.

Functional Definition of a Regulatory Sequence Conferring Hormonal Response

Poly(A)+RNA from cell clones transfected with twelve different LTR-tk plasmids was analyzed. The structure of the plasmids is shown in Figure 3. They lack

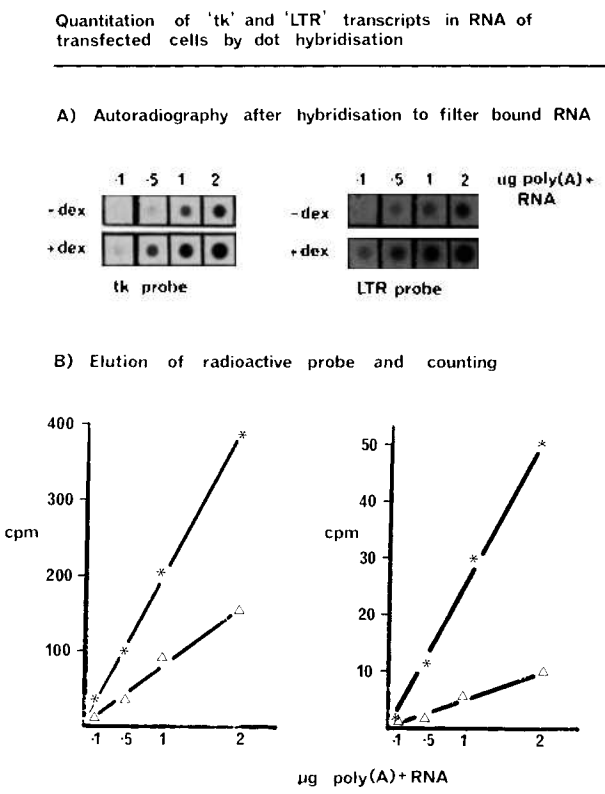

Fig. 2. Poly(A)+RNA from L-cells transfected with p 2.6 and grown in the absence and presence of dexamethasone was prepared. 0.1, 0.5, 1, and 2 μg poly(A)+RNA were dotted onto a nitrocellulose filter as described in Methods and hybridised to a tk probe (2.0-kb Pvu II fragment [14] nick-translated to a specific activity of 5×10^7 cpm/μg) or to a LTR probe (0.6-kb Pst I fragment comprising 400 nucleotides of the 3′ end of the LTR region and about 200 nucleotides of proviral gag sequences [3], specific activity of nick-translated DNA 5×10^7 cpm/μg). The hybridisation signals were visualised by autoradiography (A) for 1 day (tk probe) and for 7 days (LTR probe). The signals were eluted and counted as described in Methods (B) and the slopes of the curves obtained were used as a measure of induction of RNA as listed in Figure 3.

increasing sizes of DNA fragments from the LTR region. The base pairs of LTR remaining in the various plasmids and the nucleotides preceding the viral RNA cap site are listed. Plasmid 2.6, from which the deletions were made, can be transcribed into LTR and tk RNA, and both regions of the chimeric gene are hormone inducible. Deletion of nucleotides from the 5′ end of the LTR comprising the "open reading frame" region (p 5–5, 8–5, 10–3, 12–3, 13–13) show the same properties as the original p 2.6 (Fig. 3). The induction factor (+dex/−dex) observed with the LTR probe is between 5 and 6. Induction measured with a molecular probe specific for the tk region is 2 to 3. The difference in inducibility is probably due to the higher expression of the tk gene in the absence of hormone when compared to the transcription of LTR sequences. Transcription and induction can be measured in cells transfected with p 4′–3 (202 nucleotides upstream of cap site present) but transcription from the LTR ceases when the deletion extends to 136 nucleotides upstream of the

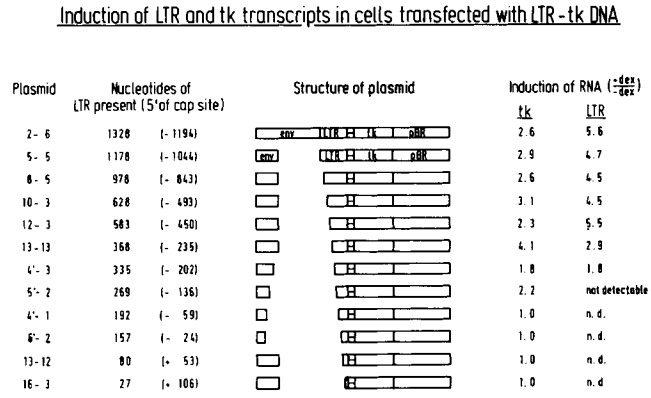

Fig. 3. Poly(A)+RNA from 12 cell clones transfected with the plasmids listed was prepared after growth in the absence and presence of dexamethasone. The LTR- and tk-specific transcripts were quantitated as described in Figure 2, and the ratios of the amount of RNA found in the presence and absence of dexamethasone are shown. The structures of the plasmids used in the transfection experiments are shown schematically in a linear representation. The regions deleted (env and LTR sequences) are left blank. The nucleotides of LTR sequences present in the individual plasmids are listed. The nucleotides present which precede the viral RNA initiation site are also shown. The viral cap site is located 134 nucleotides from the 3′ end of LTR.

viral RNA initiation site (p 5′-2). tk RNA, however, still is inducible in this transfectant. No LTR transcription and hormone inducibility is detected in cells transfected with plasmids having deletions downstream from position −59 of the viral RNA cap site (p 4′-1, 6′-2, 13-12, and 16-3). tk RNA is transcribed but does not respond to dexamethasone. This analysis shows that the DNA region located between 59 and 202 nucleotides upstream from the viral cap site harbors the signals responsible for transcription of LTR RNA and hormonal inducibility. The promoter function and the regulatory potential of the MMTV LTR are closely linked within this 143-nucleotide sequence.

DISCUSSION

Several genes have been investigated for sequences located 5′ of the RNA cap site, which influence transcription in vivo [10–12]. Two effects have been observed when deletions were introduced into the 5′ flanking regions: Modulator sequences, which govern the efficiency of gene transcription in vivo, have been found in the H2A histone gene of sea urchin [10], the iso-1-cytochrome c gene of yeast [21], the thymidine kinase gene of herpes simplex virus (HSV) [12] and the rabbit β-globin gene [11]. A second effect mediated by sequences located upstream of the structural gene is the selection of the correct 5′ termini. Deletions which leave the TATA box intact can still alter the site of RNA initiation in vivo [11]. Although the presence of modulator sequences has been established the factors operating on these signals have not yet been identified.

A LTR-tk chimeric gene has been used to functionally define the nucleotide sequence within the MMTV LTR, which is responsible for hormonal control. The

LTR-tk gene is composed of a right (3′) LTR of an exogenous provirus cloned from the DNA of a mammary tumor cell line. About 200 nucleotides derived from the cellular DNA integration site are still present between the LTR and tk regions. The LTR and its short flanking sequence is linked to the tk gene at an Eco RI site located 77 nucleotides upstream from the tk mRNA cap site. The tk promoter is still functional and tk expression is not dependent upon transcription originating in the LTR. Cells with a tk+ phenotype can be selected in the absence of glucocorticoid hormone and a functional LTR promoter. A 1.3-kb tk mRNA is transcribed and can be detected in these cells [7]. Hormonal induction of transcripts starting in the LTR and proceeding through the tk region, however, can be measured in the tk+ cells containing a functional LTR promoter upon addition of dexamethasone. These chimeric LTR-tk mRNA molecules are 1.9 kb in length [7]. Successive removal of nucleotide sequences from the 5′ region of the MMTV LTR showed that the 960 nucleotide "open reading frame" region can be entirely deleted without effect upon LTR transcription and hormone induction. A short sequence of 202 nucleotides upstream of the viral RNA cap site was shown to be sufficient to fulfill these functions. Further deletion of nucleotides located upstream of the viral cap site (up to −136) resulted in the inactivation of the LTR promoter and LTR transcription was abolished. Surprisingly, cells transfected with this plasmid still showed inducibility of tk transcripts. This observation could be explained if the hormone induction effect is not restricted to transcripts originating in the LTR but can act over a distance and affects transcripts starting at the tk mRNA cap site. Deletion of nucleotides to the −59 position with respect to the viral RNA cap site also abolished hormonal induction of tk transcripts. The finding of identical concentrations of tk transcripts made in the absence and presence of dexamethasone in cells transfected with p 4′-1, 6′-2, 13-12, and 16-3 argues for the precision of the RNA quantitation procedure. The analysis of the transcription of the deletion mutants of the chimeric LTR-tk gene shows that the viral promoter region and the signal-conferring hormonal inducibility are overlapping and located in a short stretch of nucleotides preceding the viral RNA cap site.

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