Functional redundancy of octamer elements in the mouse mammary tumor virus promoter

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ABSTRACT

The promoter of mouse mammary tumor virus contains three overlapping sequence elements related to the octamer consensus (ATGCAAAT) that are largely contained within two 10 bp direct repeats (CTTATG-TAAA) separated by a 2 bp spacer between 60 and 39 relative to the start of transcription. Gel electrophoresis mobility shift competition assays demonstrate that the most distal of these octamer-related elements is recognized by a protein that also binds to the most proximal element, while the central octamer-related element is not efficiently recognized. Transient transfection assays with altered promoters reveal that the portion of the 10 bp repeat that is not related to the octamer consensus appears not to be important for transcription. The distal and proximal octamer-related elements are, at least to some extent, functionally redundant. Complete deletion of one element has little or no effect on promoter activity so long as certain spacing constraints among remaining promoter elements are maintained. Systematic variation of such spacing reveals a cyclic effect on promoter activity corresponding to the periodicity of B-form DNA, suggesting functional interactions between proteins bound to adjacent sites.

INTRODUCTION

Promoters of genes transcribed by RNA polymerase II are, in general, composed of multiple elements, each of which serves as a binding site for a specific, *trans*-acting transcription factor (for a review, see (1–3)). Consistent with this general organization, the promoter of the retrovirus mouse mammary tumor virus (MMTV) contains at least four distinct elements that have been defined by mutational analysis and identified as sites recognized by sequence-specific DNA binding proteins (4,5). These elements include a binding site for the transcription factor nuclear factor-1 (NF-1) from 77 to 63, three sequences related to the octamer element which are almost completely contained within two exact 10 bp repeats between 60 and 39, the TATA box at about 30, and an element located 3' of the transcription initiation site between +4 and +10, which we have shown is

a binding site for a protein we have termed initiation site binding protein (ISBP) (Fig. 1).

Transcription from the MMTV promoter is subject to both positive and negative modulation. Several classes of steroid hormones, including glucocorticoids (6,7), progestins (8,9), androgens (8) and mineralocorticoids (10) induce transcription (for a review, see (11)). MMTV sequences that mediate hormone responsiveness are located immediately 5' of the promoter between about 200 and 80 (12–15), and these sequences, termed the hormone response element (HRE), contain multiple binding sites for steroid hormone-receptor complexes (16–21). The MMTV long terminal repeat also contains a mammary gland-specific enhancer element that, at least in part, determines the tissue specificity of transcription of proviral DNA (22–24). MMTV promoter activity in the absence of hormone induction is selectively repressed by a distal negative regulatory element (dNRE), located between 427 and 364, that is able to function at multiple positions in both orientations with respect to the promoter (25–28). Promoter repression mediated by the dNRE is enhanced by a promoter-proximal negative regulatory element (pNRE) (28,29), located near 160 between two adjacent binding sites for steroid hormone receptor proteins (17).

The basal promoter of MMTV must in some way respond to the positive and negative signals of the HRE and NRE. Mutations in any of the sequence elements of the MMTV promoter affect both basal and steroid hormone-induced transcription, and hormone induction does not make transcription independent of any of the DNA-binding transcription factors required for basal promoter activity (4).

Of particular relevance to the present study are the three MMTV octamer-related sequences contained within two 10 bp direct repeats (Fig. 1). The most distal of these octamer-related sequences is a 7-out-of-8 match to the consensus octamer (ATGCAAAT), while the central and most proximal of the octamer-related elements are 6-out-of-8 matches to the consensus. Altering this entire region of the MMTV promoter has a more severe effect on both basal and glucocorticoid-induced transcription than changing any single octamer-related element (4), suggesting that, at least to some extent, these elements may be functionally redundant. We present here additional evidence supporting this idea. We demonstrate specific protein recognition

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Figure 1. Structure of MMTV LTR, promoter, and octamer-related sequences. The LTR is depicted at the top with the transcription initiation site (+1) indicated by the arrow. The positions of the hormone response element (HRE, striped box), proximal negative regulatory element (pNRE, black box within the HRE), and distal negative regulatory element (dNRE, stippled box) are indicated. Elements of the basal MMTV promoter, including the TATA box, octamer-related elements, and recognition sequences for nuclear factor 1 (NF1) and initiation site binding protein (ISBP), are shown. The position and sequence of MMTV octamer-related elements is depicted at the bottom; the distal (dOct), central (cOct) and proximal (pOct) octamer-related elements are underlined, and two direct 10 bp repeats are indicated by arrows.

of the distal and proximal (but not the central) MMTV octamer-related elements and show that a single octamer-related sequence, in proper context with other MMTV promoter elements, is sufficient for wild-type levels of transcription. We also demonstrate that this context is important in that alterations in spacing between MMTV promoter elements affect transcription in a manner suggesting interactions between proteins bound to these elements.

MATERIALS AND METHODS

Plasmid constructions

Plasmids pLSwt and pLS(-64/-57) have been previously described (4). These plasmids contain MMTV LTR sequences between 363 (RsaI) and +133 (PvuII) (Fig. 1) linked to the coding sequences for bacterial chloramphenicol acetyl transferase (CAT) and the SV40 early poly (A) addition signal. pLS(-64/-57) contains a BglII linker that replaces wild-type sequences between -64 and -57. Both plasmids contain deletions in pBR322-related sequences that have been shown to inhibit gene expression in transient transfection assays (30).

pGTL1 has been described previously (4). It is identical to pLSwt with the exception that several restriction sites outside the MMTV-CAT transcription unit have been altered. pGTL5, pGTL6, pGTL9, and pGTL10 were derived from pGTL1 by oligonucleotide-mediated, site-directed mutagenesis by the method of Kunkel *et al.* (31) as described in detail (4). The structures of these plasmids were confirmed by sequencing double

stranded DNA (32) as previously described (4) and are depicted in Fig. 3. Regions containing the sequenced portion of the MMTV promoter were subcloned after site-directed mutagenesis to assure that the desired mutations were the only alterations in the plasmid sequences.

Plasmids containing spacing alterations within the MMTV promoter were derived from pLS(-64/-57). pNFOct+4 was constructed by digestion of the unique BglII site in pLS(-64/-57) followed by a Klenow fill-in reaction. pNFOct+14 was constructed by the insertion of a self complementary oligonucleotide, 5'd(GATCCAGGATCCTG)3' into the unique BglII site of pLS(-64/-57), introducing a unique BamHI site contained within the oligonucleotide. pNFOct+7A was constructed from pLS(-64/-57) by insertion of the BamHI linker 5'd(CGGATCCG)-3' into the unique BglII site which had been modified, after digestion with BglII, by partial fill-in with the Klenow fragment of DNA polymerase I in the presence of dGTP and dATP followed by mung bean nuclease digestion to generate a blunt end. pNFOct+7B was constructed by digestion of the unique BamHI site of pNFOct+14 followed by deletion of 5' overhang sequences by treatment with mung bean nuclease and recircularization with DNA ligase. pNFOct+11 was constructed by a Klenow fill-in reaction of the unique BamHI site of pNFOct+7A. pNFOct+7A was also used to construct pNFOct+3 by linearizing pNFOct+7A at the unique BamHI site followed by deletion of 5' overhang sequences by treatment with mung bean nuclease and recircularization with DNA ligase. All constructions were verified by sequencing doublestranded DNA (32) as previously described (4).

pID(-60/-50) was an internal deletion generated from pNFOct+14. pNFOct+14 was digested at its unique BamHI site and subsequently treated with mung bean nuclease and recircularized with DNA ligase. The sequence was confirmed by sequencing doublestranded DNA (32) as previously described (4).

Transfection assays

Mouse Ltk⁻ cells (33) were grown at 37°C in Dulbecco's Modified Eagle (DME) medium (Gibco) supplemented with 10% fetal bovine serum (FBS; KC Biological) in a 5% CO₂, watersaturated atmosphere.

Plasmids used in transfection assays were isolated by the alkaline extraction procedure (34) and purified by CsCl gradient centrifugation and gel filtration chromatography. Gel filtration removed impurities that affected transient CAT expression, and reproducibility of transfection results between different preparations of the same plasmid was markedly increased by including this step.

Transient transfection assays were performed by two methods. Some transfections were by the DEAE-dextran-dimethylsulfoxide shock procedure (35) as previously described in detail (36) except that, on the day prior to transfection, approximately 5×10^5 cells were seeded onto 100 mm tissue culture dishes. Other transfections were performed using DNACalcium phosphate precipitates (37). In some cases pRSVbgal (38) was cotransfected with the MMTV-CAT constructions, and bgalactosidase expression in cell extracts was measured as a monitor of transfection efficiency. CAT enzyme activities in extracts of transfected cells were determined as previously described (36) and were quantitated by direct liquid scintillation counting after separation of acetylated products by thin layer chromatography or by use of a Betascope Model 603 Blot Analyzer (Betagen,

Waltham, MA). CAT activities were normalized to total protein in the extract, or, in experiments in which pRSVbgal had been cotransfected, to β -galactosidase expression measured by hydrolysis of onitrophenylbDgalactoside (Sigma) (39). Both methods of normalization resulted in comparable levels of transcription from mutated MMTV promoters relative to the wild-type promoter normalized in the same manner.

Gel electrophoresis mobility shift assays

Nuclear extracts of Ltk⁻ cells were prepared with a slight modification of the method of Dignam (40) as previously described (41).

Oligonucleotides used as probes and competitors were synthesized with an Applied Biosystems DNA Synthesizer by Oligonucleotide Synthesis Services, Department of Biochemistry & Biophysics, Texas A&M University. Probes were labeled by filling in 5'-overhangs of annealed, complementary oligonucleotides with the Klenow fragment of DNA polymerase I and [α -³²P]dATP. Overhangs of competitors were made blunt in a similar manner using unlabeled dNTPs.

The binding buffer for the gel electrophoresis mobility shift assay contained 50 mM TrisHCl (pH 7.9), 1 mM EDTA, 1 mM DTT, 12.5 mM MgCl₂, 100 mM KCl, 20% glycerol, 67 mg/ml poly dI-dC, and competitor DNA as indicated in the legend to Fig. 2. ³²P-labeled DNA (approximately 10 fmol) was incubated with 10 mg of nuclear extract protein for 15 min on ice in a total volume of 15 ml and loaded on a 4% polyacrylamide gel containing 0.1 M Tris-borate (pH 8.3), 2 mM EDTA, and 10% glycerol. The running buffer was the same as the gel buffer except glycerol was omitted. After electrophoresis, the gel was dried and exposed to film (Kodak XAR-5) at 70°C with an intensifying screen.

RESULTS

Recognition of individual MMTV octamer-related elements by a nuclear protein

We have previously demonstrated protein binding to a DNA fragment containing all three MMTV octamer-related sequences (4). To determine whether this region of the promoter contains more than one binding site for a nuclear protein, we synthesized three double-stranded oligonucleotides that correspond to different portions of this region (Fig. 2A). The Oct-A oligonucleotide contains MMTV sequences from -66 to -41 and includes the distal (dOct) and central (cOct) octamer-related elements. The Oct-B oligonucleotide contains MMTV sequences from -54 to -31 and includes the central (cOct) and proximal (pOct) octamer-related elements. The Oct-C oligonucleotide contains MMTV sequences from -59 to -35; this oligonucleotide was synthesized with transversions at positions -57, -56, -55, -40, -39, -38, and -36 in order to isolate cOct from the dOct and pOct elements. To assess competition for protein binding between MMTV octamer-related sequences and a consensus octamer (ATGCAAAT), an oligonucleotide containing a consensus octamer (Oct-con) was synthesized; sequences immediately flanking the consensus were derived from the immunoglobulin heavy chain promoter (42). Gel electrophoresis mobility shift experiments with each of these oligonucleotides, as well as with an oligonucleotide containing no octamer-related sequences (random) were performed with nuclear extracts from Ltk⁻ cells to assess protein binding.

Figure 2. Protein binding to individual MMTV octamer-related elements assessed by gel electrophoresis mobility shift assay. **A.** Oligonucleotide probes and competitors. MMTV octamer-related elements are depicted as in Fig. 1. The sequences of oligonucleotides OctA, OctB, and OctC are shown with alterations from the MMTV wild-type sequence indicated. The sequences of the Octcon oligonucleotide, containing a consensus octamer (bold letters) in an immediate context derived from the immunoglobulin heavy chain promoter, and a random oligonucleotide containing no octamer-related elements are also shown. **B.** Gel electrophoresis mobility shift assays. ³²P-labeled OctA was incubated with nuclear extract from Ltk⁻ cells (10 mg of protein) in the presence of poly dI-dC (67 mg/ml), and the products are displayed as an autoradiogram after electrophoretic separation of free DNA from protein-DNA complexes C1 and C2. Competitor DNAs used to assess specificity of complex formation were OctA (lanes 3–5), the random oligonucleotide (lanes 6–8), Octcon (lanes 11–13), OctB (lanes 14–16), and OctC (lanes 19–21). Lanes 2, 10, and 18 contained no competitor, and lanes 1, 9 and 17 contained no nuclear extract protein. Competitors were present at 5-fold (lanes 3, 6, 11, 14 and 19), 50-fold (lanes 4, 7, 12, 15, and 20), or 100-fold (lanes 5, 8, 13, 16, and 21) molar excess over the concentration of the labeled probe.

Representative results of these experiments are shown in Fig. 2B, in which Oct-A served as the labeled probe. Two retarded bands indicative of protein-DNA complexes were observed. The more prominent band (labeled C1) results from binding to a specific protein based on its sensitivity to competition with Oct-A (lanes 3–5) and relative resistance to competition with the random oligonucleotide (lanes 6–8). The less prominent retarded band of slightly higher mobility (labeled C2) was not effectively competed by any of the DNAs tested, indicating that it results from nonspecific binding. OctB was an effective competitor; the amount of C1 complex observed decreased with increasing OctB (lanes 14–16). This competition between Oct-A and Oct-B could be the result of the significant overlap between these oligonucleotides. However, Oct-C, which contains all of the sequences shared by OctA and OctB, was a very poor competitor

Figure 3. Structure and transcriptional activity of MMTV promoters containing consensus octamer elements. **A.** Structure of mutated promoters. MMTV octamer-related elements are depicted as in Fig. 1, and mutations introduced in pGTL5, pGTL7, pGTL9, and pGTL10 are indicated. **B.** CAT expression from mutated promoters. Plasmids containing wild-type and mutated promoters were transiently transfected into Ltk⁻ cells, and CAT expression was assessed both in the presence (filled bars) and absence (open bars) of the synthetic glucocorticoid dexamethasone. CAT activity was separately normalized to that obtained from the wild-type MMTV promoter transfected under the same conditions. Dexamethasone resulted in approximately 25-fold induction of CAT activity with the wild-type promoter. These results represent the average of 3–5 transfections with a standard error of less than 20% of the mean.

(lanes 19–21). The observed competition between OctA and OctB is therefore likely to be due to the dOct and pOct elements in OctA and OctB, respectively. The cOct element in OctC does not appear to be recognized by the protein(s) present in the C1 complex. Consistent with the idea that the octamer-related elements are important for the observed protein–DNA complex formation, Octcon was also an efficient competitor, decreasing the C1 complex with increasing concentration (lanes 11–13). While it appears that OctA is a stronger competitor than OctB in Fig. 2B, this difference was not reproducibly observed.

Comparable results were obtained in similar experiments when OctB and Octcon were utilized as probes; specific complexes of mobility indistinguishable from C1 were observed that were sensitive to competition with the other oligonucleotides containing octamer-related sequences with the exception that OctC was a generally poor competitor. None of our experiments revealed significant differences in competition by OctA, OctB, or Octcon. An OctC probe also formed a comparable complex, albeit of much weaker intensity, and this complex was sensitive to competition by all of the DNAs containing octamer-related elements (data not shown).

These results suggest that a protein that recognizes the consensus octamer motif also binds to the MMTV dOct and pOct elements with roughly equal affinity and that the MMTV cOct element is bound much more weakly by this protein.

Figure 4. Structure and transcriptional activity of pID(–60/–50). **A.** Structure of pID(–60/–50). Wild-type and pID(–60/–50) sequences are shown with the dOct and pOct elements underlined and the NF1 binding site and TATA box shaded. **B.** CAT expression from pID(60/50). Plasmids were transiently transfected into Ltk⁻ cells, and CAT expression was assessed both in the presence (filled bars) and absence (open bars) of the synthetic glucocorticoid dexamethasone. CAT activity was separately normalized to that obtained from the wild-type MMTV promoter transfected under the same conditions. Dexamethasone resulted in approximately 25-fold induction of CAT activity with the wild-type promoter. These results represent the average of 6 transfections with a standard error of less than 20% of the mean.

MMTV promoters with consensus octamer elements

The octamer-related elements in the MMTV promoter are not identical to the octamer consensus sequence. It was therefore of interest to determine whether alteration of these elements to match the consensus would affect MMTV promoter function. Oligonucleotide-mediated, site-directed mutations were introduced into the MMTV promoter such that all three octamer-related elements matched the octamer consensus. These changes were made in the context of a CAT reporter plasmid (pGTL1 (4)) containing MMTV LTR sequences from –363 (RsaI) to +133 (PvuII) (Fig. 1); this allowed for promoter activity to be assessed by transfection assay in the presence and absence of glucocorticoids as we have previously described (4). Transfection of the construct containing all consensus octamer elements (pGTL5) resulted in CAT expression about 50% of that obtained from the wild-type promoter both in the presence and absence of the synthetic glucocorticoid dexamethasone (Fig. 3B). This decrease in CAT expression was also observed in additional transfections performed at different DNA concentrations (data not shown). The 5 alterations introduced into pGTL5 resulted in destruction of the perfect 10 bp repeat in this region of the MMTV promoter, altering the CTT that begins the second repeat to CAA (Fig. 3A). To determine whether this alteration in the 10 bp repeat was the cause of the 2-fold decrease in MMTV promoter activity in pGTL5, we constructed a similar plasmid in which only the distal and proximal octamer-related element retained the wild-type sequence and therefore maintained the 10 bp direct repeat (pGTL7). Transfection of pGTL7 resulted in CAT expression essentially identical to that obtained from pGTL5, indicating that the modest decrease in promoter activity observed with these constructs is due to the alterations in the

octamer-related elements. As a further test of the relevance of the 10 bp repeats in MMTV transcription, we constructed two additional promoters, each of which contained a point mutation in the portion of the 10 bp repeat that is not octamer related (pGTL9 and pGTL10). Transfection of both of these plasmids resulted in CAT expression essentially identical to that obtained from the wild-type promoter (Fig. 3B).

Deletion of the dOct element

Our previous linker-scanning analysis demonstrated the importance of the octamer-related elements in MMTV transcription (4). Gel electrophoresis mobility shift and transient transfection assays described above indicate that there are two protein recognition sites in this region of the promoter, corresponding roughly to the dOct and pOct octamer-related elements. Transcriptional activity of promoters containing linker-scanning mutations suggested that, at least to some extent, these octamer-related elements are functionally redundant; mutations in a single element had a rather modest effect on transcription relative to a disruption of this entire region of the promoter. To directly test the activity of a MMTV promoter containing only one of the two octamer-related elements that appear to be protein recognition sites, we assayed a MMTV promoter carrying a deletion between 60 and 50 (pID(60/50)). The sequences deleted from this promoter contain the dOct element (Fig. 4A). Transfection of pID(60/50) resulted in near wild-type levels of CAT expression in the presence of dexamethasone and CAT expression approximately 70% of wild-type in the absence of hormone (Fig. 4B).

Spacing changes between NF1 and octamer-related elements

The spacing between the pOct element that remains in pID(−60/−50) relative to the NF1 binding site is within 1 bp of the position normally occupied by the dOct element in the wild-type promoter (Fig. 4A). Thus, although the pID(−60/−50) carries an 11 bp deletion, the relative spacing between adjacent MMTV promoter elements is essentially identical to that in the wild-type promoter. To determine whether this spacing is important in MMTV promoter activity, we used one of our previously described (4) linker-scanning mutations (pLS(−64/−57)) to construct a series of promoters in which the nucleotide spacing between the NF1 binding site and the MMTV octamer-related elements was altered (Fig. 5). pLS(−64/−57) retains relatively high transcriptional activity both in the presence and absence of hormone induction (144% and 55% of wild-type promoter activity, respectively) and conveniently provided a BglII restriction site between the NF-1 element and the MMTV octamer region (Fig. 5). Using the BglII restriction site in pLS(−64/−57), seven different promoters were constructed that increased the spacing between the NF1 and distal octamer-related elements by 3 to 14 bp. Transfection of these constructs into Ltk[−] cells revealed a cyclic effect on promoter activity both in the presence and absence of hormone. As the NF1 site is separated from the MMTV octamer region, transcriptional activity is reduced, with the lowest level of CAT expression resulting from a spacing increase of 4–7 bp. When the separation between the sites was further increased to 9–11 bp, promoter activity was restored to wild-type levels. Further separation to 14 bp again resulted in a decrease in promoter activity. It is not likely that the specific sequences inserted are the cause of the alterations in transcriptional activity. Two different insertions of 7 bp resulted in comparably decreased CAT expression both in the presence

Figure 5. Structure and transcriptional activity of MMTV promoters containing spacing alterations between the NF1 site and octamer-related elements. Sequences of promoters are shown with the spacing changes between the NF1 site and octamer-related elements indicated. All spacing alterations were constructed in the context of pLS(−64/−57), which was used as the no insertion control in these experiments. Plasmids were transiently transfected into Ltk[−] cells, and CAT expression was assessed both in the presence (right) and absence (left) of the synthetic glucocorticoid dexamethasone. CAT activity was separately normalized to that obtained from pLS(−64/−57) transfected under the same conditions. Dexamethasone resulted in approximately 25-fold induction of CAT activity with the pLS(−64/−57) promoter. These results represent the average of 2–7 transfections with a standard error of less than 20% of the mean.

and absence of hormone, and the sequences immediately adjacent to the NF1 and octamer-related elements are similar in promoters of both low and high transcriptional activity (compare +7A with +11).

DISCUSSION

Distal and proximal octamer-related elements contribute to MMTV promoter activity

Our results strongly implicate two octamer-related elements as important in MMTV transcription. Previous experiments defined a mutation-sensitive region of the promoter between 59 and 38 which was shown to be recognized by a nuclear protein (4). Here we have demonstrated that this region contains two protein binding sites which correspond to the locations of the dOct and pOct octamer-related elements. OctA and OctB oligonucleotides, each of which contains only a single one of these elements, can be recognized by a common nuclear protein as demonstrated by gel electrophoresis mobility shift competition assays, while a third oligonucleotide, OctC, which contains all of the sequences common to both OctA and OctB, is not efficiently recognized by this protein (Fig. 2). The Oct-C oligonucleotide also contains a third octamer-related element, cOct (Fig. 1), which is apparently not efficiently recognized by the protein that binds to the dOct and pOct elements since Oct-C is not an effective competitor for protein binding to either OctA or OctB (Fig. 2).

The octamer-related elements of the MMTV promoter are almost entirely contained within two 10 bp direct repeats (C-TTATGTAATA) separated by a 2 bp spacer. The CTT sequence that begins these repeats and is not a part of the octamer-related elements appears not to be important in MMTV promoter function. pGTL5 and pGTL7, which differ only in that the CTT of the second repeat has been altered to CAA, have essentially

identical transcriptional activities in transient transfection assays. Furthermore, pGTL9 and pGTL10, which differ from the wild-type promoter only in containing a point mutation within the CTT of the first and second repeats, respectively, retain wild-type promoter activity (Fig. 3).

The presence of two protein binding sites within MMTV octamer-related elements was suggested by the results of Brüggemeier *et al.* (43). These authors utilized gel electrophoresis mobility shift assays to demonstrate that a probe containing the entire MMTV octamer region could generate two specific protein-DNA complexes which were interpreted as corresponding to complexes with either one or two octamer-related sequences occupied. Consistent with this interpretation, probes containing mutations in either the dOct or pOct element formed only one of the two complexes, presumably corresponding to that with only a single octamer-related sequence occupied. Our experiments confirm and extend these results. We have demonstrated that a common protein recognizes both binding sites within this region of the promoter and that the cOct octamer-related element apparently is not part of the sequences recognized, even though it, like the pOct element, is a 6-out-of-8 match to the consensus octamer.

The results of Brüggemeier *et al.* (43) suggested that the MMTV octamer-related elements are important only for hormone-induced transcription. This interpretation was based on a lack of effect of mutations in MMTV octamer-related sequences on basal transcription when assayed in an *in vitro* transcription system, while the same mutations had a dramatic effect on progesterone receptor-induced transcription *in vitro* and on glucocorticoid and progesterone-induced transcription *in vivo*. These authors were not able to assess basal activity of the MMTV promoter in their transfection assays. All of our transfection results, using an assay system in which basal activity of the MMTV promoter can be assessed, indicate that the MMTV octamer-related elements are required for optimal levels of both basal and steroid hormone-induced transcription. Similar to the results of Brüggemeier *et al.* (43), we have observed only a small effect of any of our octamer mutations on basal transcription *in vitro* (20–30% decrease) (J. Pierce, M. Kim, C. Bral, and D.O. Peterson, unpublished observations), even though the transcription extracts contain octamer-binding activity. It is possible that the *in vitro* systems lack an additional activity required for the oct elements to function in the absence of receptor activation, that the step in transcription initiation facilitated by the octamer-binding protein(s) is not rate determining in the *in vitro* systems, or that chromatin structures not present in DNA assayed *in vitro* are a necessary prerequisite for the maximal effect of the oct elements.

The protein(s) that mediate(s) the effect of the octamer-related elements on MMTV transcription cannot be definitively identified. Oct1, an octamer-binding protein expressed in a wide variety of tissues (44), can recognize the MMTV octamer-related elements. This has been demonstrated using partially purified Oct1 (43) as well as with purified recombinant protein expressed in bacteria (M. Huang, J. Steinke, M. Kim, and D.O. Peterson, unpublished observations). These observations make it likely that Oct1 is the protein involved. However, we have not observed increased MMTV promoter activity upon addition of recombinant Oct1 to *in vitro* transcription reactions. In addition, if Oct1 plays a role in MMTV promoter activity, it is somewhat unexpected that alteration of the MMTV octamer-related elements to match the consensus would result in the decrease in promoter activity

that we have observed with pGTL5 and pGTL7 (Fig. 3). In the absence of a cell line deficient in Oct1 expression into which appropriate Oct1 expression plasmids can be introduced or of any transcriptional response to partially purified or recombinant Oct1 *in vitro*, a definitive statement concerning Oct1 activity with the MMTV promoter cannot be made.

Functional redundancy of MMTV octamer-related sequences

Linker-scanning mutations that alter only a single octamer-related element reduce transcription substantially less than comparable mutations in other MMTV promoter elements, while more extensive alterations that eliminate all octamer-related sequences severely affect promoter activity (4). These observations previously led us to speculate that the multiple octamer-related elements in the MMTV promoter are, at least to some extent, functionally redundant. Here we provide additional evidence for this idea. The region of the MMTV promoter that contains the octamer-related elements contains two binding sites for a single protein as demonstrated by the cross competition between oligonucleotides containing the dOct and pOct elements (Fig. 2B). Deletion of one of these binding sites to generate pID(–60/–50) had little or no effect on MMTV promoter activity (Fig. 4).

The spacing between the MMTV NF1 binding site and the pOct element remaining in pID(–60/–50) is within 1 bp of the spacing between the NF1 site and the dOct element in the wild-type promoter (Fig. 4A). Maintaining the relative spacing of these elements was undoubtedly a key factor in the near wild-type promoter activity observed with pID(–60/–50). A series of promoters in which this spacing was systematically altered revealed substantial differences in transcriptional activity. Transcription decreased as the spacing was increased by an amount near half of a helical turn of B-form DNA, was near wild-type at a spacing change near a full helical turn, and was decreased again when the spacing change was near 1.5 helical turns (Fig. 5). A simple interpretation of these results is that spacing alterations that place protein binding sites on opposite sides of the DNA helix with respect to the wild-type orientation decrease promoter activity by disrupting protein–protein interactions.

These experiments do not directly address the question of which of the DNA-binding proteins associated with the MMTV promoter might interact. Because the cyclic effect on transcription was observed both in the presence and absence of glucocorticoid induction, the interacting elements must be required for both basal and hormone-induced transcription, and it is therefore unlikely that the glucocorticoid receptor protein is involved. It is tempting to speculate that proteins bound to elements immediately adjacent to the site of spacing alterations, namely NF1 and octamer-binding protein, are the interacting species. However, the spacing changes also affect the distance between the NF1 site and the TATA element, and interactions involving the basal transcription apparatus or ISBP cannot be excluded by our experiments.

Our results are reminiscent of one of the first systematic studies of spacing alterations between pol II promoter elements performed with the SV40 early promoter (45) in which mutations that alter the spacing between the TATA element and a series of binding sites for the transcription factor Sp1 were shown to affect transcription in a cyclic fashion. Mutations that altered the spacing by half a helical turn reduced transcription while mutations of a full helical turn partially or completely restored transcription.

Possible role of octamer-related elements in MMTV infectivity

MMTV is the causative agent of mammary carcinomas in many strains of mice, and viral infection occurs largely through expression of virus particles in lactating mammary tissue and passage through the milk to nursing pups (for reviews see (46–48)). This milk-borne route of infection is dependent on the immune system (49), and viral infection is thought to occur first in circulating lymphocytes (50). One potential role of MMTV octamer-related elements may be to mediate MMTV transcription in B lymphocytes early in infection via the B cell-specific octamer-binding protein Oct2, although it appears that Oct2 expression is not in itself sufficient for high levels of MMTV transcription in cells of the B lineage (51). It will be of interest to determine the detailed role of Oct2 in transcription of MMTV in B cells.

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REFERENCES

- Maniatis, T., Goodbourn, S. and Fischer, J.A. (1987) *Science*, **236**, 1237–1245.
- Saltzman, A.G. and Weinmann, R. (1989) *FASEB J.*, **3**, 1723–1733.
- Jones, N.C., Rigby, P.W.J. and Ziff, E.B. (1988) *Genes Dev.*, **2**, 267–281.
- Toohy, M.G., Lee, J.W., Huang, M. and Peterson, D.O. (1990) *J. Virol.*, **64**, 4477–4488.
- Pierce, J., Fee, B.E., Toohy, M.G. and Peterson, D.O. (1993) *J. Virol.*, **67**, 415–424.
- Ucker, D.S. and Yamamoto, K.R. (1984) *J. Biol. Chem.*, **259**, 7416–7420.
- Ringold, G.M., Yamamoto, K.R., Tomkins, G.M., Bishop, J.M. and Varmus, H.E. (1975) *Cell*, **6**, 299–305.
- Cato, A.C.B., Henderson, D. and Ponta, H. (1987) *EMBO J.*, **6**, 363–368.
- Cato, A.C.B., Miksic, R., Schtz, G., Arnemann, J. and Beato, M. (1986) *EMBO J.*, **5**, 2237–2240.
- Cato, A.C.B. and Weinmann, J. (1988) *J. Cell Biol.*, **106**, 2119–2126.
- Gnzburg, W.H. and Salmons, B. (1992) *Biochem. J.*, **283**, 625–632.
- Ponta, H., Kennedy, N., Skroch, P., Hynes, N.E. and Groner, B. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 1020–1024.
- Hynes, N., Van Ooyen, A.J.J., Kennedy, N., Herrlich, P., Ponta, H. and Groner, B. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3637–3641.
- Majors, J. and Varmus, H.E. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 5866–5870.
- Lee, F., Hall, C.V., Ringold, G.M., Dobson, D.E., Luh, J. and Jacob, P.E. (1984) *Nucleic Acids Res.*, **12**, 4191–4206.
- Von Der Ahe, D., Janich, S., Scheidereit, C., Renkawitz, R., Schtz, G. and Beato, M. (1985) *Nature*, **313**, 706–709.
- Scheidereit, C. and Beato, M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 3029–3033.
- Scheidereit, C., Geisse, S., Westphal, H.M. and Beato, M. (1983) *Nature*, **304**, 749–752.
- Payvar, F., Defranco, D., Firestone, G.L., Edgar, B., Wrange, Ö., Okret, S., Gustafsson, J.Å. and Yamamoto, K.R. (1983) *Cell*, **35**, 381–392.
- Govindan, M.V., Spiess, E. and Majors, J. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5157–5161.
- Geisse, S., Scheidereit, C., Westphal, H.M., Hynes, N.E., Groner, B. and Beato, M. (1982) *EMBO J.*, **1**, 1613–1620.
- Lefebvre, P., Berard, D.S., Cordingley, M.G. and Hager, G.L. (1991) *Mol. Cell. Biol.*, **11**, 2529–2537.
- Mink, S., Härtig, E., Jennewein, P., Doppler, W. and Cato, A.C.B. (1992) *Mol. Cell. Biol.*, **12**, 4906–4918.
- Mok, E., Golovkina, T.V. and Ross, S.R. (1992) *J. Virol.*, **66**, 7529–7532.
- Morley, K.L., Toohey, M.G. and Peterson, D.O. (1987) *Nucleic Acids Res.*, **15**, 6973–6989.
- Hsu, C.-L.L., Fabritius, C. and Dudley, J. (1988) *J. Virol.*, **62**, 4644–4652.
- Mink, S., Ponta, H. and Cato, A.C.B. (1990) *Nucleic Acids Res.*, **18**, 2017–2024.
- Lee, J.W., Moffitt, P.G., Morley, K.L. and Peterson, D.O. (1991) *J. Biol. Chem.*, **266**, 24101–24108.
- Langer, S.J. and Ostrowski, M.C. (1988) *Mol. Cell. Biol.*, **8**, 3872–3881.
- Peterson, D.O., Beifuss, K.K. and Morley, K.L. (1987) *Mol. Cell. Biol.*, **7**, 1563–1567.
- Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) *Methods Enzymol.*, **154**, 367–382.
- Chen, E.Y. and Seeburg, P.H. (1985) *DNA*, **4**, 165–170.
- Kit, S., Dubbs, D.R., Piekarski, L. and Hsu, T.C. (1963) *Exp. Cell Res.*, **31**, 297–312.
- Birnboim, H.C. and Doly, J. (1979) *Nucleic Acids Res.*, **7**, 1513–1523.
- Lopata, M.A., Cleveland, D.W. and Sollner-Webb, B. (1984) *Nucleic Acids Res.*, **12**, 5707–5717.
- Toohy, M.G., Morley, K.L. and Peterson, D.O. (1986) *Mol. Cell. Biol.*, **6**, 4526–4538.
- Kingston, R.E. (1988) In Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidmann, J.G., Smith, J.A. and Struhl, K. (eds.), *Current Protocols in Molecular Biology*. Greene Publishing Associates and Wiley-Interscience, New York, pp. 9.1.1–9.1.4.
- Gorman, C.M., Rigby, P.W.J. and Lane, D.P. (1985) *Cell*, **42**, 519–526.
- Herbomel, P., Bourachot, B. and Yaniv, M. (1984) *Cell*, **39**, 653–662.
- Dignam, J.D., Martin, P.L., Shastri, B.S. and Roeder, R.G. (1983) *Methods Enzymol.*, **101**, 582–598.
- Kuo, W.L., Vilander, L.R., Huang, M. and Peterson, D.O. (1988) *J. Virol.*, **62**, 2394–2402.
- Poellinger, L., Yoza, B.K. and Roeder, R. (1989) *Nature*, **337**, 573–576.
- Brüggemeier, U., Kalff, M., Franke, S., Scheidereit, C. and Beato, M. (1991) *Cell*, **64**, 565–572.
- Staudt, L.M., Singh, H., Sen, R., Wirth, T., Sharp, P.A. and Baltimore, D. (1986) *Nature*, **323**, 640–643.
- Takahashi, K., Vigneron, M., Matthes, H., Wildeman, A., Zenke, M. and Chambon, P. (1986) *Nature*, **319**, 121–126.
- Nandi, S. and McGrath, C.M. (1973) *Adv. Cancer Res.*, **17**, 353–414.
- Nusse, R. (1988) *Trends Genet.*, **4**, 291–295.
- Moore, D.H., Long, C.A., Vaidya, A.B., Sheffield, J.B., Dion, A.S. and Lasfargues, E.Y. (1979) *Adv. Cancer Res.*, **29**, 347–418.
- Squartini, F., Olivi, M. and Bolis, G.B. (1970) *Cancer Res.*, **30**, 2069–2072.
- Golovkina, T.V., Chervonsky, A., Dudley, J.P. and Ross, S.R. (1992) *Cell*, **69**, 637–645.
- Lund, F.E. and Corley, R.B. (1991) *J. Exp. Med.*, **174**, 1439–1450.