Identification and Characterization of a Tannic Acid-responsive Negative Regulatory Element in the Mouse Mammary Tumor Virus Promoter*

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Tannic acid, which comprises polyphenolic compounds from tea leaves, suppresses the glucocorticoid-induced gene expression of mouse mammary tumor virus (MMTV) integrated into 34I cells. To investigate whether this suppression is due to promoter responsiveness to tannic acid, we performed chromatin immunoprecipitation analysis transfecting a MMTV promoter containing a chromatin immunoprecipitation expression vector into mouse fibroblast L929 cells. Deletion analysis of the promoter region revealed that a 50-base pair (bp) region located downstream of the TATA element is responsible for the suppressive effect of tannic acid. The tannic acid-sensitive suppressibility was introduced into a thymidine kinase promoter by inserting the 50-bp region into the region on the 5′-upstream side of the promoter. Detailed point mutation analyses revealed that two elements, a 13-bp element and an ACTG motif in the 50-bp region, contribute to tannic acid sensitivity and promoter repressibility, respectively. Interestingly, this repressive ACTG motif is found in the human immunodeficiency virus promoter, the activity of which is also suppressed by tannic acid (Uchiumi, F., Maruta, H., Inoue, J., Yamamoto, T., and Tanuma, S. (1996) Biochem. Biophys. Res. Commun. 220, 411–417). Furthermore, electrophoretic mobility shift analysis revealed that a protein factor(s) in nuclear extracts from L929 cells binds to the 50-bp region in a sequence-specific manner and that the amount of DNA-protein complex is increased by tannic acid treatment. Moreover, the negative regulatory sequence ACTG and the tannic acid-sensitive 13-bp element in this region were shown to be responsible for the formation of the DNA-protein complex by electrophoretic mobility shift analysis and footprint analyses. These findings suggest that the suppressive effect of tannic acid on MMTV gene expression is mediated by a protein factor(s) that binds to the negative regulatory element containing the common ACTG motif in a cooperative manner with the tannic acid-sensitive 13-bp element.

The MMTV long terminal repeat (LTR) has been used as a model system for studying the regulation of steroid-induced transcription and the conformational effect of chromatin structure on transcription (1). The chromatin structure of the MMTV LTR has been well characterized, and it has been shown that the LTR acquires a phased array of six nucleosomes (1, 2). Binding motifs for hormone-responsive elements and other elements, such as the nuclear factor I (NF-I) and octamer binding factor binding motifs, are located in the MMTV promoter region (3–5). Both the NF-I and octamer binding factor motifs are known to be positive cis-regulatory sequences for MMTV transcription of transiently introduced templates (4). However, when the motifs are stably introduced into chromatin, they do not function unless the chromatin structure of MMTV LTR is altered by hormone (glucocorticoid) action (4, 6, 7). These observations suggest that integrated MMTV gene expression is controlled by both transcription factors and chromatin structural changes in the LTR.

We previously reported that the glucocorticoid-induced gene expression of MMTV is sensitive to tannin and related compounds (8–10). Here, we have investigated a tannic acid-responsive region in the MMTV promoter by transient transfections of the MMTV promoter containing chloramphenicol acetyltransferase (CAT) expression plasmids into the mouse fibroblastic cell line L929. A 50-bp region located downstream of the TATA element was found to be responsible for the suppressive effect of tannic acid. The introduction of various mutations in the 50-bp region suggested the presence of two separate elements, a tannic acid-sensitive 13-bp element and a negative regulatory ACTG motif. Furthermore, gel mobility shift analysis showed that a protein factor(s) that binds specifically to the 50-bp region is induced into L929 cell nuclei by tannic acid treatment. The results of gel shift competition analysis using mutated-oligonucleotides suggested that the ACTG motif and the 13-bp element play important roles in the formation of the DNA-protein complex. The specific binding of the nuclear factor(s) to the ACTG motif and the 13-bp element was confirmed by footprint analysis. These results suggest that the signal produced by tannic acid treatment is transduced to the negative regulatory motif under the control of the tannic acid-sensitive element. Previously, we found a tannic acid-responsive 30-bp element in the human immunodeficiency virus (HIV) promoter (11). A comparison of the sequences of the 50-bp element in the MMTV promoter and the 30-bp element in the HIV promoter revealed the presence of a common ACTG motif. Thus, our results may provide an important clue to controlling the regulation of retroviral gene expression through the tannic acid-sensitive element along with the ACTG motif by using tannic acid related compounds.

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†The abbreviations used are: LTR, long terminal repeat; MMTV, mouse mammary tumor virus; HIV, human immunodeficiency virus; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; NF, nuclear factor; HMG, high mobility group; EMSA, electrophoretic mobility shift assay; bp, base pair.
**Experimental Procedures**

**Materials**—Tannic acid and dexamethasone were purchased from Sigma. Acetyl CoA was from Wako Chemical Co. (Japan). [3H]Chloramphenicol and [γ-32P]ATP were from NEN Life Science Products. Restriction enzymes and other DNA modifying enzymes were from Takara (Japan). Poly(dI-dC) was from Amersham Pharmacia Biotech.

**Cell Cultures**—L929 is a mouse fibroblastic cell line (12). The 341 cell line was cultured from a C3H mouse mammary carcinoma kindly provided by Dr. Gordon Hager (13, 14). These cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 2 mM l-glutamine, and antibiotics (100 IU/ml of penicillin and 100 μg/ml of streptomycin).

**Northern Blot Analysis**—Total cellular RNAs were extracted from cultured cells by the guanidine isothiocyanate cesium chloride method. Five micrograms of total RNA was fractionated in a formaldehyde-containing 1.0% agarose gel and transferred to a nylon membrane (Pall Inc.) Hybridization of this filter with a 32P-labeled 2.0-kilobase pair EcoRI-BglII fragment covering the E fragment of the MMTV gene was carried out as described previously (8, 14). The filter was washed with 0.05 SSC buffer containing 10 mM EDTA and 0.1% SDS at 90°C for 30 min and hybridized with a 32P-labeled 1.9-kilobase pair BamHI fragment covering the human β-actin coding sequence.

**Plasmid Constructs**—The pMMTVCAT plasmid was constructed by inserting the 1.3-kilobase HindIII-XhoI fragment of the LTR region (from −1175 to +140 (15)) of the MMTV gene into the SacI site of pUC00CAT (16). The pTKCAT (pBLCAT2 (17)) plasmid contains a herpes simplex virus thymidine kinase (TK) promoter. These constructs carry the bacterial CAT gene that is expressed in eukaryotic cells under the control of each promoter.

pMMTVCAT was treated with the restriction enzyme SacI, and the large fragment was excised. This fragment, which lacks nucleotides −1175 to −109 of the MMTV promoter region, was blunt ended with T4 DNA polymerase and self-ligated. The resultant plasmid was designated pMMTV1CAT. Similarly, pMMTV2CAT, from which nucleotides +90 to +140 of the MMTV LTR were deleted, was made by excising the 50-bp BglII-SalI fragment from the pMMTVCAT construct. The pM50TKCAT and pCMTKCAT plasmids contain a 50-bp sequence of the nucleotides from +90 to +140 (M50 sequence; 5′-CCCCCGAGATCGTGGCGACTGCGGCAGACGGAATCA-3′) and three tandem repeats of seven nucleotides (CM promoter) has the following sequence.

**Transcription Assay—Plasmid DNAs were transfected into L929 cells by the DEAE-dextran method (16). Adherent cells (2 × 10⁶) were treated with 0.4 ml of TBS (25 mM Tris-HCl (pH 7.4), 137 mM NaCl, 5 mM KCl, 0.6 mM Na₂HPO₄, 0.7 mM CaCl₂, and 0.5 mM MgCl₂) containing 4 μg of the reporter plasmid, and 500 μg/ml of DEAE-dextran for 30 min at room temperature. The cells were then washed with TBS to remove unadsorbed DNA and cultivated for another 48 h in Dulbecco’s modified Eagle’s medium-10% fetal calf serum. Next, dexamethasone and tannic acid were added to the culture medium. After 16 h of incubation, the cells were collected and used for the preparation of CAT samples. CAT assays and thin-layer chromatography were performed as described previously (11, 16). Briefly, cell extracts containing 100 μg of protein were incubated with [3H]chloramphenicol and acetyl coenzyme A at 37°C for 24 h. CAT activities of the reaction products were examined by thin-layer chromatography and quantified with a Fuji BAS 2000 image analyzer system (Fuji Film, Tokyo, Japan).

**DNase 1 Footprint Analysis**—DNA-protein binding reactions and DNase 1 treatments were performed as described elsewhere (18). Briefly, a double-stranded DNA probe was prepared by annealing a 22P-labeled 500-mer oligonucleotide (5′-GATCCCGGACCTGAGGATGCGAGACTGCGGCAGACGGATCA-3′) with a nonlabeled 500-mer antisense oligonucleotide (5′-GATCTGATCCGCTGGACGACACGCTGAGGATGCGAGACTGCGGCAGACGGATCA-3′). About 0.2 ng of the probe (20,000 cpm) was incubated with 48 μg of protein (nuclear extract and bovine serum albumin) and poly(dI-dC) (0, 4, or 8 μg) in binding buffer (25 mM HEPES-KOH (pH 7.9), 50 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 10% (v/v) glycerol) (binding buffer) for 30 min at room temperature. The enzyme reaction was stopped by the addition of 3 volumes of stop buffer (0.1 mM Tris-HCl (pH 8.0), 0.5 mM NaCl, 1% sodium sarcosyl, 10 mM EDTA, and 25 μg/ml calf thymus DNA). The partially digested DNA was examined for the DNA footprint by electrophoresis on a 0.8%–1.0% (v/v) polyacrylamide gel and visualized by a chemiluminescent reaction (19).

**Electrophoretic Mobility Shift Assay (EMSA)—DNA binding reactions and EMSAs were performed by a modification of a procedure described previously (16). Briefly, two complimentary synthetic oligonucleotides were annealed (see below) and labeled with [γ-32P]ATP with T4 polynucleotide kinase.

**Results**—MMTV Gene Expression by Tannic Acid—Tannin related polyphenolic compounds, especially oligomeric ellagitannins, are potent and specific inhibitors of poly(ADP-ribose) glycohydrolase (8, 9). Because de-poly(ADP-ribose)ylation of chromosomal proteins has been suggested to be involved in the initiation of MMTV transcription (8–10), we
examined the effect of tannic acid on glucocorticoid-induced MMTV gene expression in 34I cells.

Dexamethasone, a synthetic glucocorticoid, induced dramatically both the 35S and 24S (spliced form) RNAs of MMTV transcripts (Fig. 1A, top panel; compare lane 2 with lane 1). The induction of MMTV transcription by dexamethasone was potently suppressed by pretreatment with tannic acid in a dose-dependent manner (Fig. 1A, top panel, lanes 2–4). To determine the specific suppression of MMTV expression, we performed Northern blot analysis in the same filter using β-actin cDNA, and a similar experiment was performed (middle panel). The ethidium bromide-stained gel after electrophoresis is shown (bottom panel). The signal intensities of the 35S (columns 1, 4, 7, and 10) and 24S (columns 2, 5, 8, and 11) MMTV transcripts and β-actin mRNA (columns 3, 6, 9, and 12) were quantified and normalized with the signal intensities of the 18S plus 28S rRNAs. Histograms show the relative RNA levels of 34I cells treated with 100 μg/ml tannic acid for 1 h and then treated with 200 nM dexamethasone (columns 4–12) for 30 min compared with non-drug-treated 34I cells (columns 1–3). Results are shown as means and S.E. of three independent experiments.

**Fig. 1. Effect of tannic acid on MMTV gene expression.** A, total RNAs were prepared from 34I cells treated with 100 (lane 3) or 200 μg/ml (lane 4) tannic acid or without tannic acid (lanes 1 and 2) for 1 h, and then 0 (lane 1) or 200 nM (lanes 2–4) dexamethasone was added for 30 min. The total RNAs were then subjected to Northern blot analysis with a DNA probe for the MMTV gene as described in “Experimental Procedures” (top panel). After autoradiography, the filter was reprobed with a β-actin cDNA, and a similar experiment was performed (middle panel). The ethidium bromide-stained gel after electrophoresis is shown (bottom panel). B, the signal intensities of the 35S (columns 1, 4, 7, and 10) and 24S (columns 2, 5, 8, and 11) MMTV transcripts and β-actin mRNA (columns 3, 6, 9, and 12) were quantified and normalized with the signal intensities of the 18S plus 28S rRNAs. Histograms show the relative RNA levels of 34I cells treated with 100 μg/ml tannic acid for 1 h and then treated with 200 nM dexamethasone (columns 4–12) for 30 min compared with non-drug-treated 34I cells (columns 1–3). Results are shown as means and S.E. of three independent experiments.

Inhibitory Effect of Tannic Acid on MMTV Promoter Activity—Previously, we reported that the HIV promoter is negatively responsive to tannic acid (11). To examine whether the MMTV promoter is also responsive to tannic acid, the 1315-bp LTR region of the MMTV gene was inserted upstream of the bacterial CAT gene of reporter plasmid pUC00CAT. The resulting plasmid, pMMTVCAT, was transfected into L929 cells, which were then treated with dexamethasone and tannic acid, and CAT activity was examined (Fig. 2A). The CAT activity of the dexamethasone-treated cells was about 12-fold greater than that of control cells (Fig. 2A, compare lanes 4–6 with lanes 1–3). This dexamethasone-induced MMTV promoter activity was decreased to 40% by the addition of tannic acid at a final concentration of 100 μg/ml (Fig. 2A, lanes 7–9). Because tannic acid at this concentration has essentially no effect on cell viability or protein content, further analyses were performed under these experimental conditions.

To investigate whether the tannic acid-responsive element(s) is located in the MMTV promoter region, we constructed an MMTV promoter-deleted plasmid, pMMTVΔ1CAT, from which nucleotides −1175 to −109 of the MMTV promoter region were deleted. As shown in Fig. 2, the CAT activity of cells transfected with pMMTVΔ1CAT was not induced by dexamethasone. However, we noticed that the CAT activities of both dexamethasone-treated and untreated cells were markedly lowered by tannic acid treatment (Fig. 2B, compare lanes 5 and 6 with lanes 1 and 2, or lanes 7 and 8 with lanes 3 and 4). Furthermore, the negative effect of tannic acid on the CAT activity of pMMTVΔ1CAT-transfected cells was apparent and reproducible, although the CAT activity was low (Fig. 3A). We therefore consider that there is a tannic acid-responsive element(s) located within the region comprising nucleotides −109 to +140 of the MMTV promoter.

Identification of a Tannic Acid-responsive Region in the MMTV Promoter—To determine the presence of a negative tannic acid-responsive element(s) within the region comprising nucleotides −109 to +140 of the MMTV promoter, we constructed another MMTV promoter-deleted CAT expression
nuclear factor(s) interacts with the 50-bp region including the ACTG motif, we performed DNase I footprint analysis (Fig. 4).

The amount of undigested probe increased when nuclear extracts of L929 cells were added to the binding reaction. Most of the probe was protected in the presence of nuclear extracts when poly(dI-dC) was absent from in the reaction mixture (Fig. 4A, lanes 4, 7, and 10). Because 8 μg of poly(dI-dC) was enough to prevent the nonspecific binding of nuclear protein(s) to the probe (Fig. 4A, compare lanes 5, 8, and 11 with lanes 6, 9, and 12, respectively), we decided to quantify the relative signal intensities of regions I (5′-CAGACGGGAT-3′), II (5′-CAGACGGGATCGGGGCACTGCCG-3′), and III (5′-CGGTCTGCC-3′) (Fig. 4A) against that of the undigested probe in the presence of equal amounts of poly(dI-dC). As shown in Fig. 4B, the relative signal intensity of region II clearly decreased upon the addition of L929 cell nuclear extracts, whereas the signal intensities of regions I and III were essentially unaffected. These results indicate that the nuclear factor(s) binds specifically to region II.

The relatively broad sequence in this region may include both tannic acid-sensitive and tannic acid-negative response elements.

**The ACTG Motif Is a Negative Response Element**—To determine the repressive effect of the ACTG motif, we prepared ACTG motif-containing pTKCAT plasmids (pCMm5TKCAT, pCMm6TKCAT, and pCMm7TKCAT; see “Experimental Procedures”). In all cases, a reduction in CAT activity was observed in the presence or absence of tannic acid (Fig. 5 and data not shown). This repressive effect increased in relation to the number of ACTG motifs (CM fragments) inserted upstream of the TK promoter. Six tandem arrays of CM fragments in either orientation were enough to repress the TK promoter activity by about 80%. Thus, the ACTG motif is repressive by itself independent of orientation. We further confirmed the repressive effect of the ACTG motif by using point mutants of pCMm5TKCAT (Fig. 5). The CAT activities in pCMm5TKCAT- and pCMm6TKCAT-transfected cells increased by 2.5–3.5-fold over that of pCMm7TKCAT-transfected cells. It is estimated that a single ACTG motif confers about 30% repression, whereas the mutated motifs in CMm5 and CMm6 confer about 10% repression. These results are consistent with those of gel shift competition analyses, showing that the CMm5 and CMm6 probes have about one-third the competition ability of the wild-type CM probe (see below, Fig. 9A). This suggests that the decrease in repressibility seen for the point mutants is due to lower binding affinities of the nuclear factor(s) in L929 cells for the mutated ACTG motifs.

**A 13-bp Sequence Is Involved in Tannic Acid Responsiveness**—The above observations show that the ACTG motif in the 50-bp region conveys negative promoter activity in the presence of tannic acid. Because the motif by itself does not possess tannic acid sensitivity, we suspected that another sequence in region II indicated by footprint analysis may regulate negative responsiveness under the control of tannic acid. To investigate responsiveness in detail, we introduced various point mutations into the region II of the pM50TKCAT plasmid. A single nucleotide substitution introduced in the ACTG motif (pM50m0TKCAT, pM50m1TKCAT, or pM50m2TKCAT) resulted in less tannic acid-negative responsiveness (Fig. 6A). On the other hand, a point mutation introduced into region III (Fig. 4) of the 50-bp region (pM50m3TKCAT) did not affect responsiveness. Surprisingly, a single nucleotide point mutation within the 13-bp sequence immediately upstream of the ACTG motif (pM50m0G0TKCAT) disrupted the tannic acid-negative responsiveness of pM50TKCAT completely. Thus, the tannic acid-responsive sequence is located in this 13-bp sequence.
To confirm the tannic acid responsiveness, we introduced other point mutations into the 13-bp sequence (5' -CTCAG-GTCGGCCG-3'). As shown in Fig. 6B, all mutations disrupted the tannic acid responsiveness of the M50 fragment, indicating that the 13-bp sequence is responsible for the tannic acid sensitivity. Because partial disruption of sensitivity was observed in ACTG motif-mutated reporter plasmids (Fig. 6A), there may be a binding factor(s) that associates with the two separate elements, the 13-bp sequence and the ACTG motif. In other words, the two elements may crosstalk to respond negatively to tannic acid treatment.

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sible for tannic acid sensitivity and the negative response ability. Moreover, footprint analysis showed a DNA sequence (region II) responsible for the binding of a nuclear factor(s) present in tannic acid-treated L929 cells. Therefore, we examined in detail whether any nuclear factor(s) binds sequence specifically to the 50-bp region of the MMTV promoter. Nuclear extracts were prepared from L929 cells treated with dexamethasone, tannic acid, or both. The nuclear extracts were incubated with a synthetic 50-bp double-stranded DNA probe, M50, end-labeled with 32P. As shown in Fig. 7A (compare lanes 1 and 4), the complex of M50 and nuclear protein(s) from L929 cells treated with both tannic acid and dexamethasone is clearly observable in EMSA. The binding activity of nuclear protein(s) to M50 increased by 3–4-fold, although this increase did not appear when the cells were treated with dexamethasone alone (Fig. 7A, lane 2). We further tested whether the CM probe can form a DNA-protein complex with L929 cell nuclear proteins (Fig. 7A, lane 5–8). The reaction profile of the retarded bands of the 32P-labeled CM protein complex was essentially identical to that of the 32P-labeled M50 protein complex. Therefore, the shifted bands are thought to be due to a tannic acid-inducible protein factor(s) present in L929 nuclear extracts.

To examine the induction profile more precisely, we performed EMSA using the 32P-labeled M50 probe and nuclear extracts from L929 cells treated with tannic acid (100 μg/ml) for 0–24 h. Although a small amount of the 32P-M50 protein complex was detected in the reaction mixture of nuclear extracts from untreated cells, the amount of shifted 32P-M50 probe was estimated to be about 1% of the total (Fig. 7B, lane 1). The binding activity of the nuclear extracts began to increase after 2 h of tannic acid treatment and reached a plateau after a further 6 h of treatment. The binding activity was not increased by treatment with 0.05% ethanol used as the solvent for tannic acid (Fig. 7B, lanes 9–11). Therefore, we conclude that the binding activity of nuclear factor(s) to the 50-bp region (region II) is induced by tannic acid treatment.

Sequence-specific Binding of the Nuclear Factor(s) to Tannic Acid Response Elements in the 50-bp Region—To examine the sequence specificity of M50-protein complex formation, we performed competition analyses using double-stranded DNA probes M50, CM, TL, OC, and HES-1 (see “Experimental Procedures”) as competitors. As shown in Fig. 8A, both M50 and CM, which contain the ACTG motif, competed in a dose-dependent manner with the formation of the 32P-M50 protein complex. On the other hand, the TL probe, which has been shown to bind to the large fragment of replication factor C (20), did not inhibit complex formation. Similarly, no competition was observed for the other double-stranded DNA probes, OC and HES-1 fragments, which have been reported to be suppressive element motifs (Fig. 8B). These results suggest that a nuclear factor(s) from tannic acid-treated L929 cells recognizes and binds to the 50-bp region. However, the CM probe is less effective than the M50 probe in binding (Fig. 7) and competition (Fig. 8) analyses. These results indicate that both the ACTG motif and the 13-bp sequence in the 50-bp region are involved in the specific binding of a nuclear factor(s).

To confirm the requirement of the negative ACTG motif for M50 protein complex formation, we performed similar gel shift competition analyses using various mutated CM probes as competitors. As summarized in Fig. 9A, the CM probe containing the ACTG motif competed in the formation of M50-protein complex by about 30%. CM mutant probes showed only 5–6% (CMm2 and CMm3), 10% (CMm4, CMm5, and CMm6), and 20% (CMm1) reductions in the complex formation. Taking into account the data depicted in Fig. 5, these results suggest that the four-nucleotide consensus sequence 5'-ACTG-3' is responsible for at least the formation of the M50-protein complex, as well as the repressible activity.

Because not only the ACTG motif but also the 13-bp sequence are responsible for the negative response ability with tannic acid sensitivity (Fig. 6), we further examined the role of the two elements in the binding of nuclear factor(s) by the gel shift competition analysis using various mutated M50 probes as competitors (Fig. 9B). All mutations that disrupted the tannic acid sensitivity showed almost the same level of competition as the M50 competitor probe in M50-protein complex formation. These results suggest that both independent elements, the 13-bp and the ACTG motif, are required for the binding of the nuclear factor(s) and that the two elements act cooperatively through the binding protein(s).

**DISCUSSION**

So far, several negative regulatory elements have been identified in MMTV LTR (24), all located upstream of the transcription start site. Recently, Dudley and colleagues (25) found a protein (special AT-rich sequence-binding protein 1) that binds specifically to the promoter-proximal negative element. In this study, we characterized the suppressive effect of tannic acid on MMTV promoter activity and found a 50-bp tannic acid negatively responsive element located at downstream of the other negative regulatory elements. The consensus sequence for repressiveness was determined to be 5'-ACTG-3' by comparing the 50-bp region of MMTV LTR with the 30-bp region in HIV LTR previously identified as a tannic acid-responsive negative element (11). This ACTG motif is able to confer a repressive effect on the TK promoter activity in CAT assay. EMSAs show the importance of the ACTG motif for the specific binding of a nuclear protein factor(s). Point mutations introduced into the ACTG motif resulted in decreases in both the specific binding of the nuclear factor(s) and repression in the transient CAT assay. Furthermore, point mutations in the 50-bp region of CAT constructs revealed a tannic acid-sensitive 13-bp sequence just...
upstream of the ACTG motif. The binding of the protein factor(s) to this 13-bp sequence was supported by footprint analysis. The 50-bp binding activity of the protein factor(s) from L929 cell nuclear extracts was found to be inducible by tannic acid treatment. This is the first evidence that MMTV LTR contains a novel tannic acid-responsive negative regulatory element (containing the ACTG motif plus the 13-bp sequence) downstream of the previously identified negative regulatory elements (24, 25).

MMTV LTR has been well characterized as a steroid-inducible promoter (1). It locates a hormone-responsive region that contains four hormone receptor binding elements and confers responsiveness to steroid hormone action (26). Furthermore, the chromatin structure of integrated MMTV LTR has been shown to be organized into an array of at least six positioned nucleosomes (2, 15). Recently, the importance of chromatin remodeling by the glucocorticoid receptor in the transcription of the stably integrated MMTV gene was clearly demonstrated (27). The LTR region also locates other protein factor binding sites, such as the NF-I binding site (28), AP-2 element (29), and two octamer motifs (5, 30). Associations between the protein factors and these transcriptional elements and the subsequent alterations in chromatin structure by the interactions between the factors and the chromatin constructing proteins are thought to play important roles in chromosome-integrated MMTV gene expression (4–7, 15, 31).

MMTV transcription is also known to be regulated by modifications in chromosomal proteins, such as acetylation (32, 33) and poly(ADP-ribosyl)ation (10, 14). Hyperacetylation of histones induced by histone deacetylase specific inhibitors has been shown to increase chromosome-integrated MMTV and HIV-1 gene expression (32, 33). Histone hyperacetylation catalyzed by histone acetyltransferases (34) is believed to lead to allosteric changes in nucleosomal conformation that destabilize
chromatin structure (35, 36). Thus, the hyperacetylation of histones is thought to cause transcriptional activation. Furthermore, several transcriptional factors, including p300/CBP and p300/CBP-associated factor, have recently been shown to possess histone acetyltransferase activity in mammalian cells (37, 38). Previously, we reported that the induction of the MMTV mRNA is associated with a loss of poly(ADP-ribose) from the HMG 14 and HMG 17 proteins (14). Moreover, we observed that oenotein B, a specific inhibitor of poly(ADP-ribose) glycohydrolase, suppresses glucocorticoid-induced MMTV transcription in 3T3 cells (10). In this case, suppression is accompanied by the inhibition of glucocorticoid-induced endogenous de-poly(ADP-ribosyl)ation of the HMG 14 and HMG 17 proteins, which are known transcriptional activators (39, 40). Our present data clearly show that tannic acid treatment induces the ability of L929 cell nuclear extracts to bind to the 50-bp region of the MMTV LTR. Interestingly, the 50-bp region partly overlaps with the boundary of the Nuc-A position (2, 41). The specific protein factor(s) that binds to the tannic acid-responsive sequence containing the ACTG motif and the 13-bp sequence might interact with poly(ADP-ribosyl)ated HMG proteins and/or histones to affect their nucleosomal assembly systems and thereby suppress MMTV transcription. Further investigations are needed to confirm this hypothesis and to elucidate the role of the tannic acid-responsive DNA binding factor(s) that negatively regulates MMTV gene expression.

Fig. 7. Induction of MMTV 50-bp tannic acid-responsive element binding activity in L929 cells. A, the double-stranded oligonucleotide probes M50 (lanes 1–4) and CM (lanes 5–8) were 5’-end-labeled with [γ-32P]ATP using T4 polynucleotide kinase. L929 cells were treated with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) 200 nM dexamethasone in the presence (lanes 3, 4, 7, and 8) or absence (lanes 1, 2, 5, and 6) of 100 μg/ml tannic acid, and nuclear extracts were prepared. The labeled probe (approximately 10 fmol) was incubated in buffer with 2 μg of poly(dI-dC) and 1 μg of nuclear protein extract for 30 min at room temperature. The reaction mixture was electrophoresed in a 4% nondenaturing polyacrylamide gel. A band showing a shift in mobility is indicated by the arrowhead. B, nuclear extracts were prepared from L929 cells treated with 100 μg/ml tannic acid for 0, 0.5, 1, 2, 4, 8, 16, or 24 h (lanes 1–8, respectively) or prepared from cells treated with 0.05% ethanol for 0, 8, or 24 h (lanes 9–11, respectively). EMSA was performed using 1 μg of nuclear protein extract with a 32P-end labeled M50 probe and 2 μg of poly(dI-dC). A band showing a shift in mobility is indicated by the arrowhead.

Fig. 8. Sequence specific binding of a nuclear factor(s) to probes containing the ACTG nucleotide motif of the MMTV 50-bp element. A, competition analysis of 32P-M50 protein complex formation. The competitors used for EMSA were M50 (lanes 2–4), TL (lanes 5–7), and CM (lanes 8–10). Binding of the 32P-labeled M50 probe (10 fmol) and 1 μg of nuclear extract from L929 cells treated with 100 μg/ml tannic acid for 4 h and 2 μg of poly(dI-dC) was examined as described in the legend to Fig. 7A. The amounts of competitor DNA were 100 fmol (lanes 2, 5, and 8), 300 fmol (lanes 3, 6, and 9), or 1 pmol (lanes 4, 7, and 10). Lane 1 shows a binding assay without competitor. B, an experiment similar to that described in A was done using the double-stranded DNA probes M50 (closed circles), CM (closed triangles), TL (open circles), OC (open squares), and HES-1 (open triangles) as competitors. The radioactivities of the shifted band in each assay mixture formed by incubating 32P-labeled M50 with the nuclear extract of L929 cells treated with 100 μg/ml tannic acid for 4 h were quantified with a Fuji BAS 2000 image analyzer system. Results represent the averages of two independent experiments.
The integrated HIV promoter has been shown to contain a periodic array of nucleosomes, like the MMTV promoter (42). Transcriptional regulatory elements such as NF-κB, Sp-1, and TATA motifs are located in the HIV LTR (43–45). A recent study has shown that several changes in the local chromatin structure of the HIV promoter induced by NF-κB play important roles in regulating transcription from the integrated HIV provirus (46). The HS4 element in the HIV promoter has been shown to be associated with the 3′ terminus of the U5 region and to contain a cluster of potential binding sites for the AP-1, AP-3-like, DBF-1, and Sp-1 proteins (47). In addition, the nucleotide sequence of this element has been shown to be required for the control of transcription at the chromatin-organized HIV promoter (48). Like that of the MMTV promoter, the chromatin structure of the integrated HIV promoter is affected by specific transcription factors (49, 50). It is noteworthy that the negative ACTG motif found in the MMTV promoter is also present in the HIV LTR. As indicated in Fig. 6, the role of this motif by itself may be to repress the inherent activities of the proviral promoters in the absence of inducers, such as glucocorticoids and tumor necrosis factor. The factor(s) that binds to the ACTG motif that is present in both the MMTV and HIV promoters may negatively regulate MMTV and HIV gene expression by interacting with other transcription factors or chromatin-associated proteins to alter the nucleosomal conformations to transcriptionally inactive states. To clarify the molecular mechanism by which the ACTG motif represses MMTV and HIV gene expression in tannic acid-treated cells, molecular cloning of cDNAs that encode the MMTV 50-bp and HIV 30-bp element (or ACTG motif) binding protein(s) is required. Our present data support the proposal of Smith and Hager (51) that consideration of chromatin structural changes in LTR is important in understanding transcriptional regulation in vivo. We believe that the characterization of viral LTR binding proteins and their interactions with chromosomal proteins may not only clarify transcription regulatory mechanisms but also contribute to the development of therapies for retrovirus-associated diseases.

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