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The Polyomavirus Enhancer Activates Chromatin Accessibility on Integration into the *HPRT* Gene

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Recent studies suggest that enhancers may increase the accessibility of chromatin to transcription factors. To test the effects of a viral enhancer on chromatin accessibility, we have inserted minigenes with or without the polyomavirus enhancer into the third exon of the hypoxanthine phosphoribosyltransferase (*HPRT*) gene by homologous recombination and have prepared high-resolution maps of gene accessibility by using a novel polymerase chain reaction assay for DNase I sensitivity. In its native state, we find that the *HPRT* gene has low sensitivity to DNase I in fibrosarcoma cells. Insertion of the polyomavirus enhancer and *neo* reporter gene into exon 3 confers altered *HPRT* DNase I sensitivity for several kilobases on either side of the enhancer. The changes in DNase I sensitivity peak near the enhancer and decline with distance from the enhancer. The increase in *HPRT* DNase I sensitivity persisted when the tk promoter was deleted from the inserted construct but disappeared when the enhancer was deleted. These experiments identify the polyomavirus enhancer as a *cis*-acting initiator of chromatin accessibility.

Eukaryotic DNA forms a complex with the histones, which tightly fold genomic DNA into chromatin fibers. Measurements of the accessibility of nuclear DNA to nucleases such as DNase I have demonstrated that modification of chromatin packaging accompanies or precedes changes in gene transcription (15, 25, 35, 43, 46, 51, 53, 54, 59, 60). Recent genetic experiments with yeast histone mutants support a causal role for chromatin accessibility in gene regulation by showing that reductions in histone stoichiometry or modification of histone H4 lead to marked transcriptional activation of many genes (7, 19–21, 24, 33). Moreover, chromatin reconstitution experiments have demonstrated that some enhancer- or promoter-binding proteins can effectively compete with histones to specifically counteract histone-mediated repression and increase local accessibility to transcription factors (2, 8, 9, 13, 26, 36, 52, 55–57) and to DNase I (12).

The increased chromatin accessibility of active regions is characterized both by DNase I-hypersensitive sites that form locally over enhancer and promoter sequences (11, 18), and by regional or domain DNase I sensitivity, which extends over many kilobases of chromatin (1, 23, 25, 43, 54). Recent studies (10, 15, 16, 60) have suggested that some enhancers may mediate long-range domain effects on chromatin accessibility.

We wished to determine the extent to which an enhancer-containing gene cassette might perturb a domain of chromatin into which it was inserted. In order to facilitate probing chromatin regions around the insert and to preclude position effect variations inherent to random integration, we have used the technique of site-directed mutagenesis to target various constructs by homologous recombination into a known single-copy genomic locus (45). The human X-linked *HPRT* gene (34) in HT1080 cells with the male karyotype of one X chromosome was chosen for these targeting experiments because (i) no *HPRT* enhancer has been found (34), (ii) native DNase I sensitivity was reported to be low (28, 58), and (iii) mutant clones with a single disrupted *HPRT* gene can be selected by their resistance to the nucleotide analog 6-thioguanine (45). After minigene insertion, we mapped DNase I sensitivity of the surrounding chromatin with a novel polymerase chain reaction (PCR)-based assay (16). We find that integration of a polyomavirus enhancer-containing insert leads to a marked increase in *HPRT* DNase I sensitivity which centers around the enhancer and is lost when the enhancer is deleted. Since the increases in DNase I sensitivity persist even when the promoter is deleted, these experiments point to the polyomavirus enhancer element as an activator of domain DNase I sensitivity.

MATERIALS AND METHODS

Recombinant DNA. Southern blots containing *Hin*dIII-restricted DNA from control and targeted HT1080 cells were probed with ^{32}P-labeled inserts from *HPRT*-containing plasmids pG2PE and pG2PG (28). Plasmid pG2PE has a 0.6-kb *Pst*I-*Eco*RI fragment found downstream of exon 3 (probe B, adjacent to the targeted insertion site; see Fig. 1A), while pG2PG has a 1.1-kb *Eco*RI-*Bgl*III fragment from downstream of exon 6 (probe A, Fig. 1A).

PCR primers. For the DNase I-PCR assay, PCR primers were designed to probe the middle portion of the β-globin gene, seven regions of the *HPRT* locus (see H05 to H57 in Fig. 1A), and the *neo* gene. All PCR primers were 26 to 30 bases long, and opposing *HPRT* primers spanned 329 to 402 bp, while the globin primers spanned 465 bp. The nucleotide primer sequences are given below along with their map locations (in parentheses).

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Globin A	5'GGAGACCAATAGAAACTGGGCATGTGGAGA3'	(55314 to 55343)
Globin B	5'CGATCCTGAGACTTCCACACTGATGCAATC3'	(55750 to 55779)
Neo A	5'CCTTGCTCCTGCCGAGAAAGTATCCATCAT3'	(1058 to 1087)
Neo B	5'CAGCAATATCACGGGTAGCCAACGCTATGT3'	(1399 to 1428)
H05A	5'GTCCTAGAAATGTAATCCTGCCCTAG3'	(4711 to 4736)
H05B	5'CCAAGGCAACTCTTCTCTGCTGATCTGGTT3'	(5011 to 5040)
H15A	5'CATCAGCAGCTGTTCTGAGTACTTGCTATT3'	(14940 to 14969)
H15B	5'GCCAGACATACAATGCAAGCATTCAATACC3'	(15282 to 15311)
H18A	5'TCCTTTTAGGAATTGCTGTTGGGACTTGGG3'	(17013 to 17042)
H18B	5'TCACACTGATTTTGTTTCATCCACAGTGTC3'	(17334 to 17363)
H22A	5'TACCTAGTGCCTAGATGGGAAATTGCCTGG3'	(20209 to 20238)
H22B	5'TGGGTTCAAGCCAGCCTCCCGTCTTGACTT3'	(20529 to 20558)
H26A	5'TGGGGCCTGCTTGAATGTTGAGAGAATGAC3'	(24401 to 24430)
H26B	5'CTCTAGGTAGCAATAAGAACTGCAGCATGG3'	(24716 to 24745)
H35A	5'TGTGATTGTAGGACTGAGGGCCCGTTTTCT3'	(33961 to 33990)
H35B	5'CATCAGAAACATCATGGCTGGAGACTTGTG3'	(34334 to 34363)
H57A	5'CTTGCCCGCCTGATGAATGCTCATCCCGAA3'	(56000 to 56029)
H57B	5'TAAGCATTCTGCCGACATGGAAGCCATCAC3'	(56325 to 56354)

Gene targeting. The *neo* gene under the control of the thymidine kinase (tk) promoter and the polyomavirus enhancer in pH*neo*-Py were derived from a 1,083-bp *Xho*I-*Sal*I fragment of the Stratagene expression vector pMC1*neo*. To prepare an enhancerless mutant (pH*neo*-ΔE), we deleted a 278-bp fragment containing the polyomavirus enhancer and substituted a 260-bp portion of the bacterial chloramphenicol acetyltransferase coding region (Fig. 1B). To prepare a promoterless mutant (pH*neo*-PyΔP), we deleted a 148-bp fragment containing the tk promoter from the pMC1*neo* vector. In order to establish cell lines carrying these cassettes, HT1080 cells were grown in medium containing hypoxanthine-aminopterin-thymidine to select against spontaneous *HPRT* mutants. A total of 10^7 cells were electroporated with 20 to 40 μg of targeting vector and plated in medium without hypoxanthine-aminopterin-thymidine. After 5 days of recovery, 2 μg of 6-thioguanine per ml was added to select for *HPRT* mutants. Resistant clones were screened for correctly targeted inserts by Southern blotting. Depending on the construct, 5 to 25% of the isolates had correctly targeted inserts. Two independent isolates were then chosen for detailed DNase I analysis.

Cell culture, isolation of nuclei, and DNase I digestions. Human HT1080 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's Nutrient F-12 medium (no. 8900; Sigma) supplemented with bovine calf serum (Hazleton Laboratories). The cells were washed, scraped from the plate, and collected in ice-cold phosphate-buffered saline. The cells were pelleted and resuspended in cold buffer A (10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$) along with 0.4% Nonidet P-40 and 1 mM *p*-(chloromercuri)benzenesulfonic acid as described previously (14). After 5 min, the resulting lysate was centrifuged at 2,000 × *g* for 5 min to pellet the nuclei. The nuclei were resuspended in buffer A at a concentration of 800 μg/ml (assayed by dilution into 2 M NaCl and 5 M urea) and stored on ice briefly. Five 360-μl aliquots of each sample were preincubated at 37°C for 5 min. The nuclei were digested for 3 min at 37°C with various concentrations of DNase I (D-5025; Sigma). Under these conditions, optimal preferential DNase I sensitivity was typically obtained at a level of 1 μg of nuclease per reaction. Reactions were stopped by adding sodium dodecyl sulfate (SDS) and EDTA to final concentrations of 0.4% SDS and 20 mM EDTA.

DNA isolation, Southern blotting, and hybridization. DNA was purified from the nuclei by phenol-chloroform extractions as previously described (48) and digested overnight at 37°C with 2 U of various restriction enzymes per μg of DNA. The restricted DNA was subjected to either PCR or Southern blot analysis. Blot analysis was carried out by standard methods (42) as previously described (15). The blots were hybridized to *HPRT* intron probes A and B and autoradiographed at −80°C for 5 days.

PCR analysis of DNase I-treated samples. DNase I-digested DNA samples (0.5 μg each) were analyzed as before (16) by using duplex PCR (4, 5, 39, 41, 49) with primers for the β-globin control and *HPRT* test probes. Because a single cut within a probed sequence will prevent amplification of the sequence, coamplification of an active and an inactive region allows detection of preferential sensitivity within the active domain in comparison to the inactive one (16). Amplification was carried out at three standard temperatures (94, 57, and 72°C) for 22 cycles in a Perkin-Elmer Cetus thermocycler. The final reaction products were electrophoresed on 6% polyacrylamide gels, stained with ethidium bromide, visualized under 254-nm UV light, and photographed with Tmax film (Kodak) (negative, 4 by 5 in. [10.16 by 12.70 cm]). The PCR bands in the film were quantitated by scanning with a Hoefer densitometer, and the areas under the peaks were integrated with the aid of a microcomputer. Equivalent results were obtained if quantitation was carried out by incorporating radioactively labeled nucleotides and counting the dissected PCR bands by Cerenkov radiation. Normalized DNase I sensitivity (*S*) values were calculated from the equation $S_H = \log (H_D/H_U)/\log (G_D/G_U) \times T$, where *H* and *G* are *HPRT* and globin band intensities for the undigested ($_U$) or digested ($_D$) samples and *T* is the size ratio of the globin to *HPRT* bands.

DNase I-hypersensitive sites. The presence of DNase I hypersensitivity sites within our *HPRT* probes would confound our analysis of domainwide DNase I sensitivity. However, the only observed DNase I-hypersensitive site in active *HPRT* chromatin is located at the 5' promoter (34), which is remote from our sensitivity probes. In the chroma-

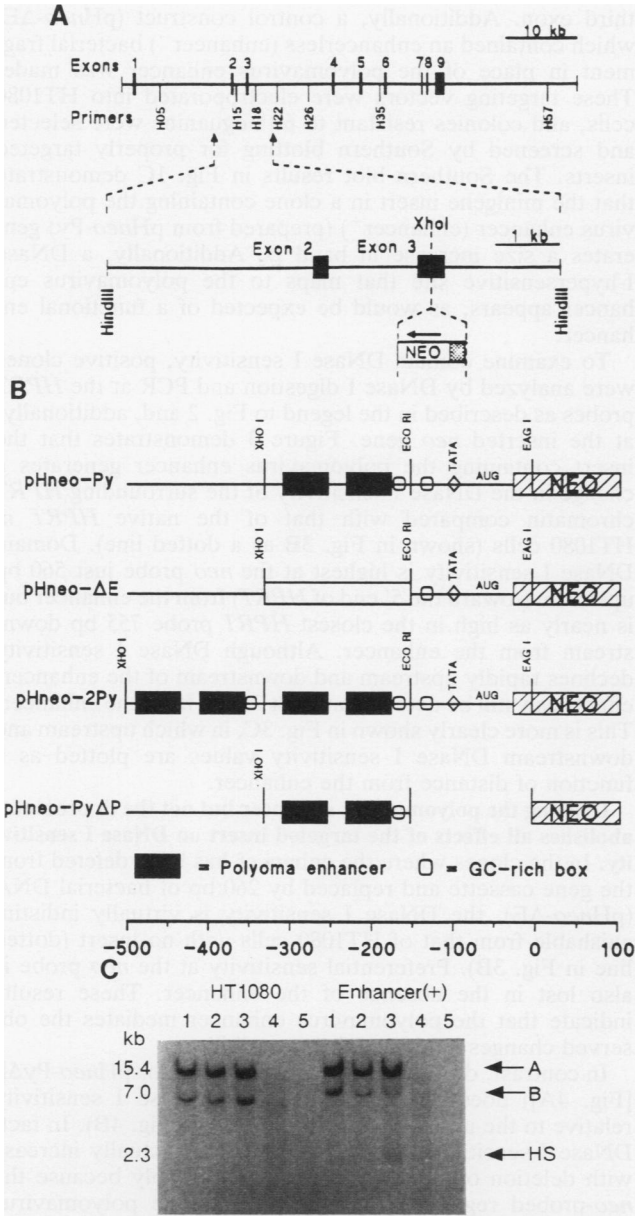

FIG. 1. Targeting *neo* minigenes into *HPRT*. (A) The upper map shows a sequenced 57-kb region of the human *HPRT* gene with its nine exons (34) and the seven PCR primer pairs used in DNase I sensitivity analysis (H05 to H57). The enlargement of exons 2 and 3 shows the 6.9-kb *Hin*dIII *HPRT* fragment used to make the targeting vectors. The minigene constructs containing the herpes simplex virus tk promoter and the *neo* gene were inserted into the *Xho*I site of exon 3. (B) The pH*neo*-Py vector has the polyomavirus enhancer adjacent to the tk promoter. To prepare the enhancerless mutant pH*neo*-ΔE, a 278-bp fragment containing the enhancer was deleted and a 260-bp fragment from the bacterial chloramphenicol acetyltransferase gene was substituted. To generate pH*neo*-PyΔP, a 148-bp *Eag*I-*Eco*I fragment was deleted from pH*neo*-Py to remove the upstream GC box and the TATA box (30). To prepare pH*neo*-2Py, the polyomavirus enhancer region was doubled. (Because the pMC1*neo* construct consists of a tandem repeat of the polyomavirus enhancer sequences, a single enhancer actually contains two copies and a double contains four copies of the enhancer element.) Scale at bottom indicates base pairs. (C) DNase I digestion of control (HT1080) or pH*neo*-Py targeted (enhancer$^+$) nuclei is shown. Nuclei were digested with increasing concentrations of DNase I (lanes 2 to 5; lanes 1, undigested DNA). The DNA was purified, restricted with *Hin*dIII, electrophoresed on a 1.0% agarose gel, and Southern blotted. The blot was hybridized to *HPRT* probes A and B (see Materials and Methods). When hybridized to *Hin*dIII-restricted DNA, probe B hybridizes just downstream of exon 3 to detect a 6.9-kb (in HT1080) or 8.0-kb (in the cells carrying the insert) band, while probe A hybridizes near exon 6 to a 15.4-kb band. Thus, in enhancer$^+$ cells, probe B allows confirmation of the expected 1.1-kb insert. Moreover, DNase I digestion reveals a hypersensitive site (HS) that maps to the enhancer region in enhancer$^+$ cells.

tin of targeted pH*neo*-Py mutants, a second hypersensitive site has been mapped over the polyomavirus enhancer (Fig. 1B) and is located several hundred base pairs from the flanking *neo* and H18 probes.

Sensitivity of naked DNA to DNase I. To control for the possibility that some *HPRT* DNA sequences differ in native sensitivity to DNase I, purified HT1080 DNA was digested to the same size distribution as that of the nuclear DNA. This DNA was then assayed for relative DNase I sensitivity by using the PCR technique as before.

RNA isolation and RT-PCR quantitation of mRNA levels. Total cellular RNA was purified by guanidine-thiocyanate extraction (6). As a check on the quality and quantity of RNA, aliquots were denatured with glyoxal and dimethylsulfoxide (31) and electrophoresed on a 1% agarose gel and the RNA was visualized by ethidium staining and UV illumination. mRNA levels were assayed by reverse transcriptase (RT)-PCR analysis (4, 5, 41, 49). Briefly, cDNA was prepared by using RT with antisense *neo* primers (see Fig. 5A) or random oligoprimers (see Fig. 5B). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control. The cDNA was amplified for 21, 25, or 31 cycles for GAPDH, *neo*, or *HPRT*, respectively. For the *HPRT* intron probe analysis, 5 μCi of [^{32}P]dATP was added during the amplification reactions. After electrophoresis, radioactivity incorporated into the amplified bands was measured by a flatbed radiation scanner. Control experiments in which RT was left out showed that less than 10% of the signal was caused by the amplification of contaminating genomic DNA. Other control experiments showed that amplification was in the linear range of detection.

RESULTS

The *HPRT* locus has a low level of DNase I sensitivity. We first examined the native DNase I sensitivity of the *HPRT* locus in nontargeted HT1080 fibrosarcoma cells grown in hypoxanthine-aminopterin-thymidine medium to select for *HPRT* expression. Purified nuclei were subjected to DNase I digestion, and the degree of digestion along the *HPRT* gene was determined by PCR amplification of 329- to 402-bp regions (primer pairs H05 to H57, Fig. 1A). Because a single cut within a DNA strand prevents its amplification, greater digestion within a region generates a weaker band when the PCR products are fractionated electrophoretically (Fig. 2A). Relative DNase I sensitivity at each probed *HPRT* sequence was determined by coamplification with 465 bp of the nontranscribed β-globin gene and comparison of the relative intensities of the *HPRT* and globin bands. As a control for differential DNase I sensitivities in naked DNA, genomic HT1080 DNA was purified until free of nuclear protein, digested to the same size distribution as that of the DNA in nuclei, and amplified as before (bottom of Fig. 2A).

The *HPRT* band intensities from all PCR amplifications were quantitated by densitometry and divided by the values

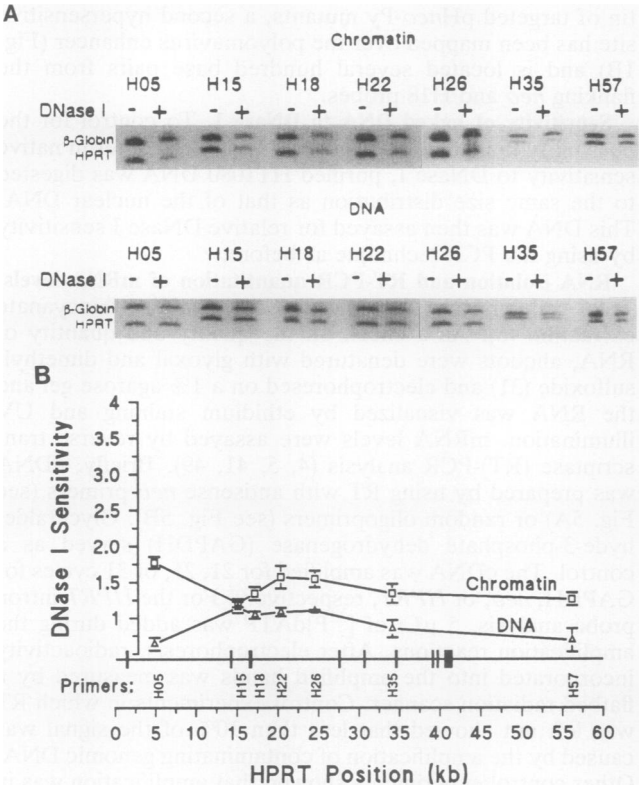

FIG. 2. DNase I sensitivity of *HPRT* chromatin and DNA from control cells. (A) To determine DNase I sensitivity of *HPRT* relative to β-globin chromatin (upper panel), multiplex PCR was carried out on 250 ng of undigested or 500 ng of digested DNA by coamplifying a β-globin probe with each *HPRT* probe (H05 to H57 in Fig. 1). To control for the DNase I sensitivity of naked DNA, multiplex PCR was also performed after digestion of purified DNA (lower panel). (B) DNase I sensitivity was quantitated by scanning bands from three to five replicate amplifications at each probed region. The mean normalized values ± the standard errors of the mean were plotted above a map of the *HPRT* domain.

obtained for the β-globin internal controls and the undigested DNA controls. These normalized *HPRT* sensitivity values were further corrected for target size and then plotted as relative DNase I sensitivity at each probed position along the *HPRT* domain. Figure 2B demonstrates that the DNase I sensitivity of naked DNA is close to 1 for each *HPRT* region probed, indicating the absence of sequence-specific sensitivity relative to globin DNA. When packaged as chromatin, however, *HPRT* DNA has nearly twofold preferential sensitivity at the 5′ end, falling to lower sensitivity over most of the rest of the gene. These values for *HPRT* DNase I sensitivity, while low compared with those of most active genes, are consistent with the earlier studies of *HPRT* DNase I sensitivity carried out in other cell lines by using the conventional Southern blot technique (28, 58).

Insertion of a minigene containing the polyomavirus enhancer alters *HPRT* chromatin. Since DNase I sensitivity of the native *HPRT* gene was low near exon 3, we tested whether integrating a transcriptionally active minigene into exon 3 would generate increased DNase I sensitivity in the adjacent *HPRT* chromatin. The minigene construct (pH*neo*-Py [Fig. 1]) contained the *neo* gene under the control of the tk promoter and the polyomavirus enhancer inserted into a 6.9-kb *Hin*dIII fragment of the human *HPRT* gene at the third exon. Additionally, a control construct (pH*neo*-ΔE) which contained an enhancerless (enhancer$^-$) bacterial fragment in place of the polyomavirus enhancer was made. These targeting vectors were electroporated into HT1080 cells, and colonies resistant to 6-thioguanine were selected and screened by Southern blotting for properly targeted inserts. The Southern blot results in Fig. 1C demonstrate that the minigene insert in a clone containing the polyomavirus enhancer (enhancer$^+$) (prepared from pH*neo*-Py) generates a size increase in band B. Additionally, a DNase I-hypersensitive site that maps to the polyomavirus enhancer appears, as would be expected of a functional enhancer.

To examine domain DNase I sensitivity, positive clones were analyzed by DNase I digestion and PCR at the *HPRT* probes as described in the legend to Fig. 2 and, additionally, at the inserted *neo* gene. Figure 3 demonstrates that the insert containing the polyomavirus enhancer generates a change in the DNase I sensitivity of the surrounding *HPRT* chromatin compared with that of the native *HPRT* in HT1080 cells (shown in Fig. 3B as a dotted line). Domain DNase I sensitivity is highest at the *neo* probe just 560 bp upstream (toward the 5′ end of *HPRT*) from the enhancer but is nearly as high in the closest *HPRT* probe 755 bp downstream from the enhancer. Although DNase I sensitivity declines rapidly upstream and downstream of the enhancer, effects can still be detected at least 2.5 kb from the enhancer. This is more clearly shown in Fig. 3C, in which upstream and downstream DNase I sensitivity values are plotted as a function of distance from the enhancer.

Deleting the polyomavirus enhancer but not the tk promoter abolishes all effects of the targeted insert on DNase I sensitivity. In the clones where the enhancer has been deleted from the gene cassette and replaced by 260 bp of bacterial DNA (pH*neo*-ΔE), the DNase I sensitivity is virtually indistinguishable from that of HT1080 cells with no insert (dotted line in Fig. 3B). Preferential sensitivity at the *neo* probe is also lost in the absence of the enhancer. These results indicate that the polyomavirus enhancer mediates the observed changes in chromatin accessibility.

In contrast, deleting the tk promoter (vector pH*neo*-PyΔP [Fig. 4A]) does not decrease *HPRT* DNase I sensitivity relative to the undeleted pH*neo*-Py levels (Fig. 4B). In fact, DNase I sensitivity of the *neo* gene may actually increase with deletion of the tk promoter, presumably because the *neo*-probed region is 148 bp closer to the polyomavirus enhancer because of the deletion. These data further point to the polyomavirus enhancer as the principal activator of enhanced DNase I sensitivity in *HPRT*-targeted cells.

Adding an additional polyomavirus enhancer does not further change DNase I sensitivity. We asked whether the effect of the polyomavirus enhancer on *HPRT* chromatin structure could be increased by incorporating two polyomavirus enhancers upstream of the tk promoter (see pH*neo*-2Py in Fig. 4B). Figure 4B demonstrates that an additional polyomavirus enhancer does not significantly change chromatin structure compared with the single-enhancer insert. These data indicate that the chromatin effects of the polyomavirus enhancer are not additive.

The polyomavirus enhancer induces a high level of *neo* transcription while transcription of the *HPRT* probed regions appears unchanged. Since *HPRT* transcription rates are typically very low (32), the detection of transcription by nuclear run-on assays is difficult. We have therefore used the highly sensitive RT-PCR assay (4, 5, 41, 49) to detect the low levels of unprocessed transcripts at each *HPRT* and *neo*

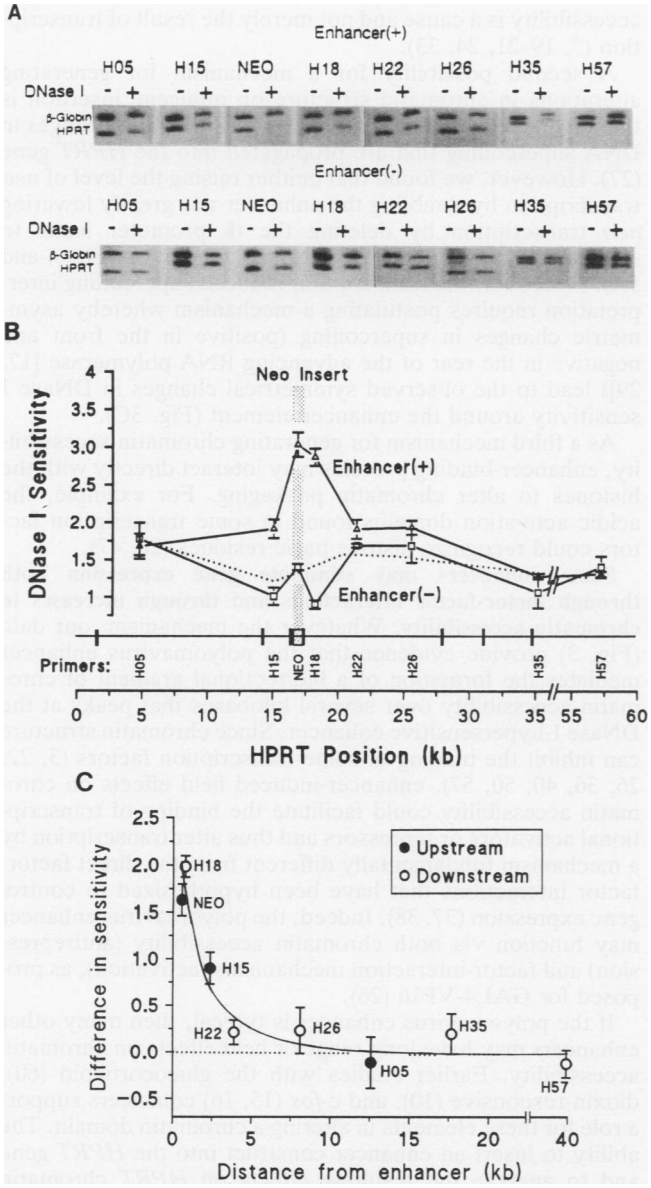

FIG. 3. DNase I sensitivity of *HPRT* chromatin from targeted cells. Nuclei were purified from *HPRT* mutants pH*neo*-Py (enhancer$^+$) and pH*neo*-ΔE (enhancer$^-$) and analyzed for DNase I sensitivity as in the experiment described in the legend to Fig. 2. (A) PCR products from the enhancer$^+$ and enhancer$^-$ clones are displayed as before. In the targeted cells, sequences within the *neo* insert (NEO) were amplified in addition to the *HPRT* probes. (B) Mean results from three to six determinations (± standard errors of the mean) for each clone were plotted. For comparison, the native DNase I sensitivity profile of control HT1080 chromatin from Fig. 2B is shown as a dotted line. (C) The difference in sensitivity between enhancer$^+$ and enhancer$^-$ chromatin was plotted as a function of distance from the polyomavirus enhancer.

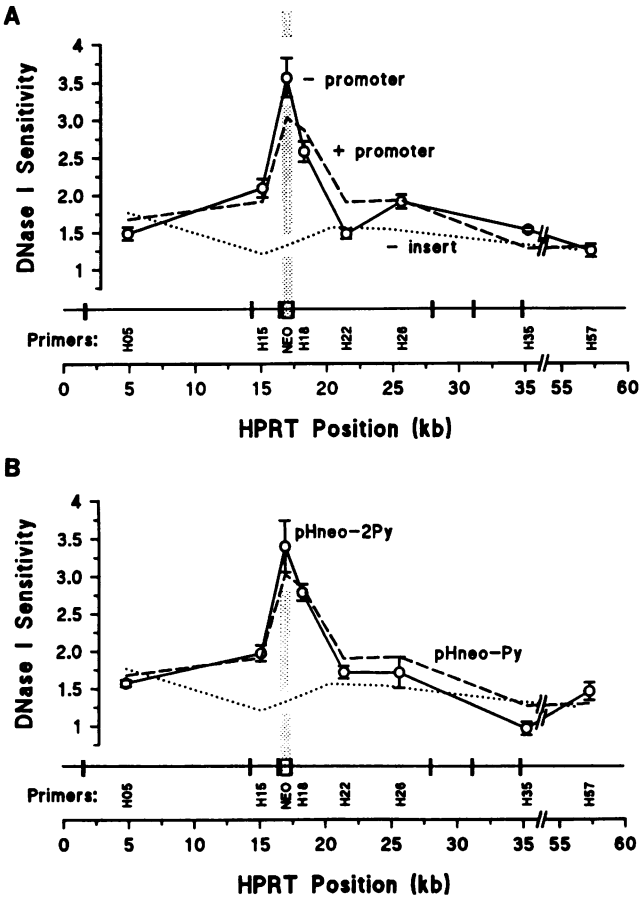

FIG. 4. DNase I sensitivity of *HPRT* with targeted minigenes containing a promoter deletion. Nuclei were purified from promoterless mutant pH*neo*-PyΔP and double-enhancer construct pH*neo*-2Py and analyzed for DNase I sensitivity as in the experiment described in the legend to Fig. 2. (A) Mean results from three determinations (± standard errors of the mean) of the promoterless mutant were plotted. (B) Mean results from three determinations of the double enhancer are shown. For comparison, the native DNase I sensitivities of control HT1080 nuclei (dotted line) and targeted HT1080 nuclei (pH*neo*-Py) from Fig. 3 (dashed line) are shown in both panel A and panel B. Dotted region, targeted minigene insert.

primer pair. The RT-PCR data in Fig. 5A (lane 2) demonstrate that the polyomavirus enhancer and the tk promoter stimulate transcription of the *neo* gene. With the double-enhancer construct (lane 3), the level of *neo* transcription is further increased. Conversely, deleting the polyomavirus enhancer (ΔPoly, lane 1) or the tk promoter (ΔPromoter, lane 4) abolishes all (lane 1) or most (lane 4) *neo* transcription.

In contrast to the *neo* gene, the data in Fig. 5B suggest that *HPRT* intron transcription is unchanged in the presence or absence of the polyomavirus enhancer. While these data cannot rule out the possibility that short, rapidly processed transcripts are induced from cryptic promoters in localized regions of *HPRT* (44), the results in Fig. 4B offer no support for an enhancement in the basal transcription of the probed regions of *HPRT*.

DISCUSSION

An enhancer-containing minigene has field effects on *HPRT* chromatin structure. We have shown that the insertion of a complete minigene construct into the third exon of *HPRT* induces a bidirectional gradient of chromatin accessibility that peaks at the enhancer. While deleting the tk promoter does not decrease *HPRT* DNase I sensitivity, deletion of the enhancer from the minigene abolishes all effects of the

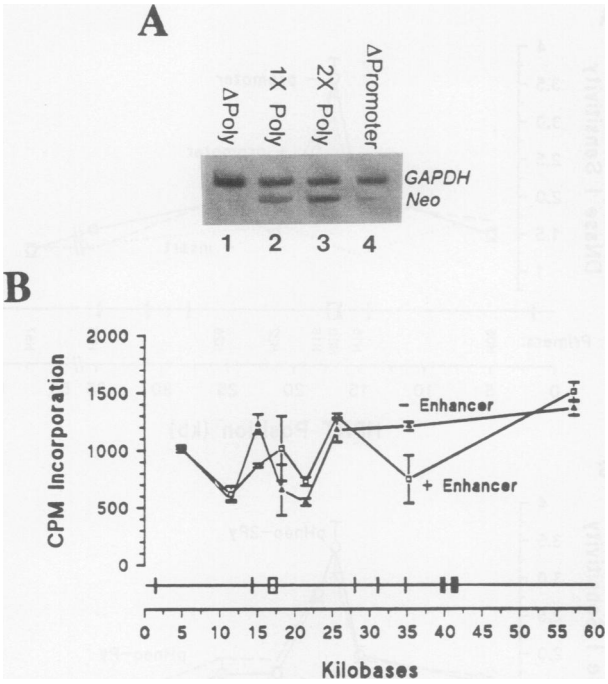

FIG. 5. RNA expression from the *neo* and *HPRT* genes of control and targeted cells. RNA from targeted cells was reverse transcribed and analyzed by quantitative RT-PCR. GAPDH, a housekeeping gene that is transcribed equally in all cell lines, served as an internal control. (A) Because *neo* is transcribed in the opposite direction from the *HPRT* gene in our constructs (Fig. 1A), we could analyze transcripts initiating from the *neo* promoter by using the antisense *neo* primer during reverse transcription. cDNA from GAPDH or *neo* was amplified for 21 or 25 cycles, respectively, and electrophoresed on a polyacrylamide gel. Lanes 1 to 4 contain pH*neo*-ΔE (ΔPoly), pH*neo*-Py (1× Poly), pH*neo*-2Py (2× Poly), and pH*neo*-PyΔP (ΔPromoter), respectively. (B) To assay for any enhancer-induced change in message at probes H05 to H57, cDNA was prepared from the RNA of enhancer$^+$ and enhancer$^-$ clones by using random oligopriming. The cDNA was amplified in the presence of 5 μCi of [^{32}P]dATP for 31 cycles by using the *HPRT* primers. After electrophoresis, radioactivity incorporated into the amplified bands was measured by a flatbed radiation scanner. Counts per minute (CPM) incorporation reflects determinations in triplicate of the RNA levels at each *HPRT* primer pair.

targeted insert on chromatin structure, implicating the polyomavirus enhancer as a required initiator of the chromatin accessibility gradients.

How might the polyomavirus enhancer affect long-range changes in *HPRT* chromatin structure? One possibility is that *HPRT* chromatin becomes more accessible to DNase I as a direct result of enhanced transcription from the *HPRT* sequences. Although we cannot completely rule out this possibility, several lines of evidence suggest that transcription is unlikely to be the explanation for altered chromatin accessibility. First, *HPRT* is transcribed at low levels without a targeted insert, and these levels do not appear to increase with minigene targeting even for those *HPRT* probes with high DNase I sensitivity (Fig. 5B). Second, previous studies have reported that DNase I sensitivity correlates with the potential for transcription rather than the rate of transcription itself (10, 15, 25, 43, 46, 51, 53, 54, 59, 60). Third, nucleosome loss in yeast histone mutants leads to derepression of certain genes, indicating that chromatin accessibility is a cause and not merely the result of transcription (7, 19–21, 24, 33).

A second possibility for a mechanism for generating alterations in chromatin structure on minigene insertion is that high levels of *neo* transcription might lead to changes in DNA supercoiling that are propagated into the *HPRT* gene (27). However, we found that neither raising the level of *neo* transcription by doubling the enhancer nor greatly lowering *neo* transcription by deleting the tk promoter leads to significant changes in *HPRT* DNase I sensitivity (Fig. 4 and 5A). Moreover, the transcription-induced supercoiling interpretation requires postulating a mechanism whereby asymmetric changes in supercoiling (positive in the front and negative in the rear of the advancing RNA polymerase [17, 29]) lead to the observed symmetrical changes in DNase I sensitivity around the enhancer element (Fig. 3C).

As a third mechanism for generating chromatin accessibility, enhancer-binding proteins may interact directly with the histones to alter chromatin packaging. For example, the acidic activation domains found in some transcription factors could recognize histone basic residues (20, 47).

Some enhancers may stimulate gene expression both through factor-factor interactions and through increases in chromatin accessibility. Whatever the mechanism, our data (Fig. 3) provide evidence that the polyomavirus enhancer mediates the formation of a bidirectional gradient of chromatin accessibility over several kilobases that peaks at the DNase I-hypersensitive enhancer. Since chromatin structure can inhibit the binding of some transcription factors (3, 22, 26, 36, 40, 50, 57), enhancer-induced field effects on chromatin accessibility could facilitate the binding of transcriptional activators or repressors and thus alter transcription by a mechanism fundamentally different from the direct factor-factor interactions that have been hypothesized to control gene expression (37, 38). Indeed, the polyomavirus enhancer may function via both chromatin accessibility (antirepression) and factor-interaction mechanisms (activation), as proposed for GAL4-VP16 (26).

If the polyomavirus enhancer is typical, then many other enhancers may have long-range or field effects on chromatin accessibility. Earlier studies with the glucocorticoid (60), dioxin-responsive (10), and c-*fos* (15, 16) enhancers support a role for these elements in altering a chromatin domain. The ability to insert an enhancer construct into the *HPRT* gene and to analyze the resulting effects on *HPRT* chromatin provides a novel method for examining the field effects of various enhancer elements and thus should help elucidate the distal chromatin effects of these important regulatory sequences.

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