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The human growth hormone locus control region mediates long-distance transcriptional activation independent of nuclear matrix attachment regions

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ABSTRACT

Expression of the human growth hormone (hGH-N) transgene in the mouse pituitary is dependent on a multicomponent locus control region (LCR). The primary determinant of hGH LCR function maps to the pituitary-specific DNase I hypersensitive sites (HS) HSI,II, located 15 kb 5′ to the hGH-N gene. The mechanism by which HSI,II mediates long-distance activation of the hGH locus remains undefined. Matrix attachment regions (MARs) comprise a set of AT-rich DNA elements postulated to interact with the nuclear scaffold and to mediate long-distance interactions between LCR elements and their target promoters. Consistent with this model, sequence analysis strongly predicted a MAR determinant in close proximity to HSI,II. Surprisingly, cell-based analysis of nuclear scaffolds failed to confirm a MAR at this site, and extensive mapping demonstrated that the entire 87 kb region encompassing the hGH LCR and contiguous hGH gene cluster was devoid of MAR activity. Homology searches revealed that the predicted MAR reflected the recent insertion of a LINE 3′-UTR segment adjacent to HSI,II. These data point out discordance between sequence-based MAR predictions and *in vivo* MAR function and predict a novel MAR-independent mechanism for long-distance activation of hGH-N gene expression.

INTRODUCTION

Matrix attachment regions (MARs) comprise discrete DNA segments dispersed throughout the metazoan genome. MARs are operationally defined as DNA sequences that are retained on the nuclear scaffold after chromatin has been selectively depleted of histones by salt extractions (1). Although well-defined structurally and biochemically (2,3 and references therein) the *in vivo* function(s) of MARs in gene expression remain to be clearly identified and tested.

MARs are postulated to serve a variety of functions in gene expression. The periodic attachment of chromatin to the nuclear scaffold may organize chromatin within the nucleus (1,4) and sequester local *cis*-acting DNA elements within specific and functionally defined nuclear subregions. Indeed, the association of RNA polymerase II with the nuclear matrix suggests that transcription itself occurs in association with these structures (2,5, reviewed in 6,7). MARs have been shown to insulate reporter transgenes from position effects (8) as well as to augment the activity of enhancer elements by extending the distance over which they can function (9,10). The latter activity has been linked to specific extension of DNA demethylation (9) and chromatin core histone acetylation (11,12) from MAR-associated enhancers to target promoters. As such, MARs are thought to subserve important functions in the context of long-distance interactions mediated between locus control regions (LCRs) and their respective target promoters (12–14).

MARs lack a unique sequence consensus. However, conserved sequence motifs and sequence compositions have been noted. These include origins of replication, A+T-rich sequences, topoisomerase II binding sites and topologically bent or kinked DNA (15). A MarFinder algorithm has been assembled based on the integration of the frequency of these determinants in a defined sequence relative to the probability of their random occurrence in that sequence (MarFinder: http://www.futuresoft.org/MAR-WIZ/). This algorithm has successfully predicted experimentally determined MARs at the β-globin, protamine, and apolipoprotein B loci (16).

The human growth hormone gene cluster is composed of five structurally-related genes arranged in a common transcriptional orientation: 5′ hGH-N, hCS-L, hCS-A, hGH-V and hCS-B 3′ (Fig. 1A). The 5′-most gene, hGH-N, is expressed exclusively in pituitary somatotropes while the remaining four genes are expressed in the syncytiotrophoblasts of the placenta (17). Activation of the hGH gene cluster in both the pituitary and placenta is dependent on an LCR. The hGH LCR is comprised of five DNase I hypersensitive sites (HS) distributed from 15 to 32 kb 5′ of the hGH-N promoter (18,19) (Fig. 1A). The two closely linked and pituitary-specific HS, HSI and HSII, are both necessary and sufficient for high-level, position-independent activation of hGH-N transgenes in mouse somatotropes (18,20,21). Understanding how HSI,II activates the hGH promoter over a span of 15 kb is of central importance to defining the mechanisms of LCR action. In the current

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study, we test whether MAR element(s) are associated with the hGH LCR.

MATERIALS AND METHODS

Sequence analysis

The 1602 bp HSI,II sequence (GenBank accession no. AF039413) was analyzed using the Internet-based MarFinder algorithm (16). A window size of 200 bp stepped at 10 bp intervals was used in the initial analysis. No sequence rules were omitted from the calculation. Sequence alignments were performed using MacVector software, version 6.5.1.

MAR assay

Cells were harvested from the pituitaries of multiple hGH/P1 transgenic adult mice as described previously (21). HeLa cells were maintained at 37°C in 5% CO_2 in DMEM, containing 10% fetal bovine serum and 1% penicillin/streptomycin, prior to harvesting. Namalwa cells were maintained at 37°C in 5% CO_2 in RPMI 1640, containing 10% fetal bovine serum and 1% penicillin/streptomycin, prior to harvesting. The preparation of nuclear matrices under isotonic conditions was adapted from the procedure of Mirkovitch *et al.* (1). Specifically, 10^7 cells were collected by centrifugation at 650 *g* for 5 min at 4°C and washed twice in 1 ml cold PBS. Cells were resuspended in 1 ml of 10 mM PIPES pH 7.8, 100 mM NaCl, 0.3 M sucrose, 3 mM $MgCl_2$, 0.5% Triton X-100, and incubated on ice for 15 min until lysis occurred. Nuclei were collected by centrifugation at 1000 *g* for 5 min at 4°C, and washed twice with 1 ml cold PBS. Nuclei were gently resuspended in 100 µl PBS, and incubated at 37°C for 20 min to stabilize the nuclear scaffolds. One milliliter of 10 mM PIPES pH 7.0, 10 mM EDTA, 2 mM KCl, 0.25 mM spermidine, 0.1% w/v digitonin and 25 mM lithium 3,5-diiodosalicylic acid (LIS) was added dropwise while vortexing gently to extract histones. Nuclear halos were collected by centrifugation at 12 000 *g* for 10 min at 4°C, and washed five times with 1 ml of 50 mM Tris–HCl pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 0.1% v/v aprotinin. Nuclear halos were resuspended in the same buffer including 400 U *Bgl*II (New England Biolabs), and incubated for 16 h at 37°C with gentle inversion. Soluble DNA (loop fraction) was separated from insoluble DNA (MAR fraction) by centrifugation at 12 000 *g* for 30 min at 4°C. The supernatant was removed to a separate tube, and the pellet was resuspended in 400 µl of the above restriction enzyme digestion buffer. Both fractions were treated with 0.1 g/l proteinase K in the presence of 1% SDS for 30 min at 37°C. Fractions were extracted with an equal volume of phenol, then with an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol. DNA was precipitated from the fractions with 1/10 vol 3 M sodium acetate and 2 vol absolute ethanol, washed with 70% ethanol and resuspended in 100 µl of 10 mM Tris pH 8.0 and 1 mM EDTA. DNA concentration was determined using a Hoefer DNA fluorometer and Hoechst 33258 dye (Sigma).

Five micrograms each of the MAR and loop fractions were resolved by electrophoresis in a 0.8% agarose gel in 40 mM Tris–acetate, 1 mM EDTA, pH 8.0 and visualized by staining in 5 µg/ml ethidium bromide. Resolved DNA was transferred to a positively-charged nylon membrane (Zetabind; Cuno Laboratory Products) by upward capillary transfer in 0.4 M NaOH using standard methods. Specific regions of DNA were detected by Southern hybridization to the nylon membrane using random-primed [α-^{32}P]dATP-labeled probes and standard procedures. All hybridizations were performed using the same blot; probe was stripped from the membranes between hybridizations by incubation with 0.2× SSC, 0.2% SDS at 95°C. Bands were visualized by autoradiography, and quantified by exposure of the hybridized blots to a storage phosphor screen with subsequent analysis by a Molecular Dynamics phosphorimager and ImageQuant software.

Nuclear halos were visualized by immobilizing nuclei from the cell lysis step onto glass slides in a cytocentrifuge (Shandon Cytospin 3) at 2000 r.p.m. for 5 min. Subsequent treatments to achieve nuclear halos were performed as above by applying the reagents directly to the slide. Nuclear halos were stained with 5 µg/ml Hoechst 33342 for 5 min, followed by a 5 min wash in dH_2O prior to visualization by fluorescence microscopy at 1000×.

Table 1. Probes employed to detect specific regions of the hGH locus

Probe	Position relative to hGH-N cap site
1	–32744 to –32198
2	–26491 to –26104
3	–18825 to –18317
4 (HSI,II)	–16345 to –14743
5	–9539 to –9111
6	–492 to +746
7	+1363 to +3666
8	+4400 to +6487
9	+6489 to +14277
10	+14300 to +16927
11	+16927 to +19112
12	+25212 to +31709
13	+38291 to +40905

Probes employed to detect specific genomic regions

The human β-globin gene was detected with a cDNA probe encompassing the exon sequences from nucleotides 62279 to 63610, GenBank accession number U01317. The mouse immunoglobulin µ (Igµ) fragment containing the enhancer-associated MARs was detected with a probe encompassing nucleotides 3395–3681, GenBank accession number J00440. The position of probes used to detect regions of the human growth hormone locus are shown in Table 1.

RESULTS

Prediction of a MAR associated with HSI,II

Previous studies have demonstrated that a 1.6 kb restriction fragment encompassing DNase I hypersensitive sites I and II (HSI,II) of the hGH LCR confers high-level and position-independent expression upon a linked hGH-N transgene in the mouse pituitary (18,20,21). HSI,II is naturally located 15 kb 5′ to the hGH-N transcription initiation site. Based on demonstrations of MAR-dependent LCR functions in other

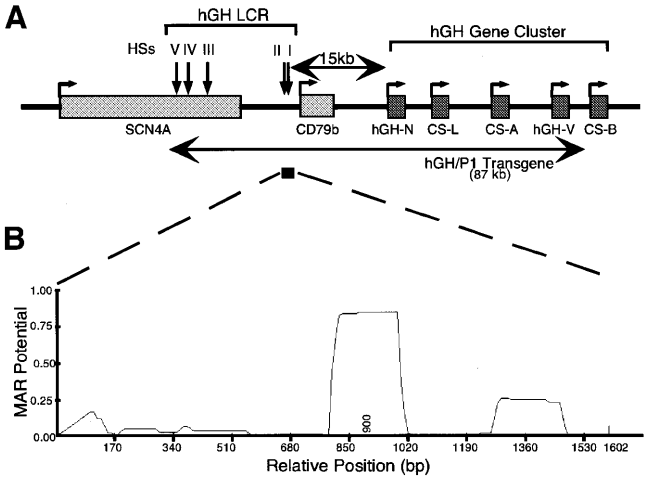

Figure 1. MarFinder analysis of HSI,II of the hGH LCR. (**A**) The hGH gene cluster and its linked LCR. A diagram of the hGH locus is shown. DNase I HS of the hGH LCR are indicated by vertical arrows. The 1.6 kb *Bgl*II restriction fragment encompassing HSI,II is shown as a black bar below the map. The genes of the hGH gene cluster are indicated as dark grey boxes, with their transcriptional orientations shown by the arrows. The linked but unrelated CD79b (Igβ) and SCN4A genes are also shown (light grey boxes). The boundaries of the hGH/P1 transgene are delimited below the map. (**B**) Analysis of MAR potential within the HSI,II region. The line graph of MAR potential score versus nucleotide position along the 1602 bp HSI,II sequence using a 200 bp window at 10 bp intervals is shown. A matrix potential of 0.6 is considered the minimum significance threshold by the program. The positions of the significant peaks are indicated on the *x*-axis.

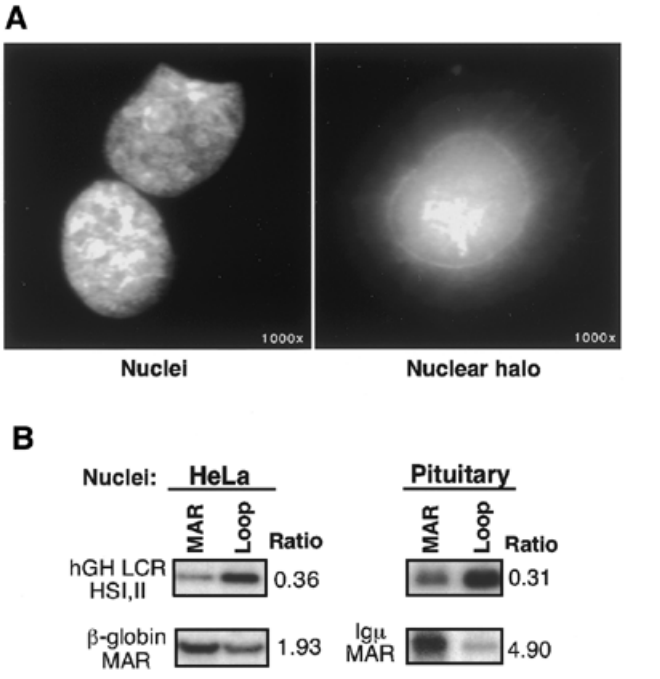

Figure 2. MAR assay of the HSI,II region. (**A**) Nuclear halo formation. Intact HeLa cell nuclei (left) and post-LIS extraction nuclear halos (right) were stained with Hoechst 33342. The image is shown at 1000× using a CCD camera. (**B**) MAR assay of HSI,II region in HeLa cells and in primary pituitary cells. The pituitary cells were harvested from a mouse line carrying an hGH/P1 transgene encompassing the hGH gene cluster and contiguous LCR (see Fig. 1A). The cell-based MAR assay was carried out as described in Materials and Methods. The frames show the region of the respective Southern blots containing the hybridization signals to the noted probes. The ratios of hybridization signal strength (MAR:Loop) is shown to the right of the respective frames.

systems (9,22), a role of MAR function in the action of HSI,II was investigated. The initial approach involved scanning the 1.6 kb sequence containing HSI,II for MAR determinants (MarFinder, http://www.futuresoft.org/MAR-WIZ/) (16). A peak of MAR potential was detected and considered to be highly significant on the basis of comparative studies (Fig. 1B). The position of this peak adjacent to the major enhancer element of the hGH LCR (HSI,II) was remarkably similar to the positioning of functional MARs next to the major enhancer element of the Igμ LCR (4,23). These data suggested that HSI,II was associated with a MAR determinant.

Verification of the MAR assay

The cell-based detection of MARs involves the removal of histones and other non-histone proteins from chromatin by LIS extraction and subsequent identification of the DNA segments attached to the nuclear scaffold. After LIS extraction, the decondensed genomic DNA projects outward from the nuclear lamina network and forms a 'nuclear halo' (Fig. 2A). Digestion of the halo preparation with a restriction enzyme solubilizes ~70% of the DNA. This released DNA, the 'loop fraction', is considered to lack direct association with the nuclear matrix. The remaining 'MAR fraction' DNA can then be extracted from the sedimented DNA/protein complex. Equal masses of DNA purified from the loop and MAR fractions are analyzed by Southern blot using a series of unique sequence probes representing sites of interest. Restriction fragments containing sequences mediating scaffold association will be enriched in the MAR fraction, and their MAR:loop hybridization signal ratio will be >1.

HSI,II lacks MAR determinants in isolated nuclei

Most MARs appear to be constitutive (15). This is true even for MARs associated with tissue-specific genes (24). Thus, the initial search for a MAR associated with HSI,II was carried out in HeLa cells (Fig. 2B). The data revealed that HSI,II sequences were enriched in the loop fraction. In contrast, the human β-globin gene in the same preparation was enriched in the MAR fraction at the 2-fold level, fully consistent with prior reports (14,24). Thus, HSI,II was not associated with MAR determinants in HeLa nuclei.

It is possible that MAR activity associated with HSI,II might be limited to nuclei of cells expressing the hGH-N gene. Since there are no human somatotrope cell lines available for study, a transgenic mouse model for the human growth hormone locus was employed. This transgenic line carries an 87 kb P1 clone (hGH/P1) encompassing the entire hGH LCR and hGH gene cluster (Fig. 1A). This hGH/P1 transgene fully recapitulates the normal human pattern of hGH-N expression (19) and LCR chromatin modification (25) in the mouse pituitary. HSI,II is formed in chromatin isolated from the pituitaries of these transgenic mice and the expression of the hGH-N gene is dependent on the presence of HSI,II function (Y.Ho, University of Pennsylvania, personal communication). Pituitaries from a representative hGH/P1 transgenic mouse line were isolated and a Southern blot of fractions from *Bgl*II-digested nuclear halos was prepared. As observed in HeLa cells, the 1.6 kb

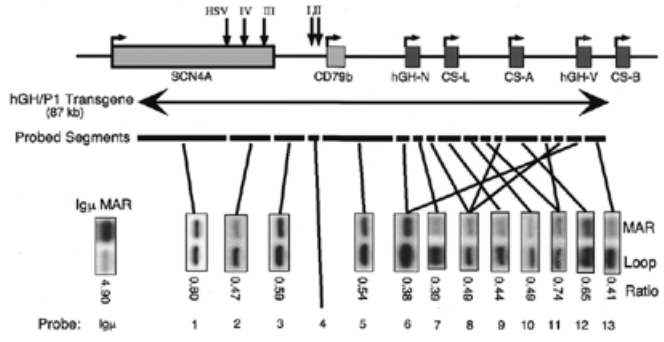

Figure 3. The hGH locus was devoid of MAR determinants. The hGH locus and hGH/P1 transgene are as described in Figure 1A. The series of contiguous *Bgl*II fragments spanning the entire region is indicated by black bar segments below the diagram. The Southern blot hybridization results with probes detecting each fragment are shown, and the corresponding MAR/Loop fraction signal ratios are indicated below the respective autoradiograms. The corresponding probes are also indicated. Cases of multiple fragments corresponding to a single hybridization signal indicate paralogous fragments of identical length that cannot be resolved by hybridization. The results for fragment 4 are shown in Figure 2B.

fragment containing HSI,II was enriched in the loop fraction (Fig. 2B). The filter was stripped and rehybridized with a probe corresponding to the MARs associated with the mouse Igμ heavy chain LCR enhancer (4,23) as a positive control for this study. In full agreement with prior reports, this region was highly enriched in the MAR fraction (~5-fold; Fig. 2B). These data suggested that the HSI,II region was devoid of MAR determinants in expressing as well as non-expressing cells.

The hGH LCR and contiguous hGH gene cluster lack MARs

The initial analysis of MARs in the hGH LCR focused primarily on the region in close proximity to HSI,II. This was based on the dominant role of these sites in hGH LCR function and on the strong prediction of MAR potential by the MarFinder algorithm. The inability to detect MAR determinants adjacent to HSI,II by biochemical assay (Fig. 2B) suggested two possibilities: either the MARs that facilitate the long-range gene activation by HSI,II are located elsewhere in the hGH LCR, or hGH LCR-mediated activation was MAR independent. To distinguish between these two possibilities, the remaining regions of the hGH LCR and adjacent hGH gene cluster were probed for MARs (Fig. 3). Since the full sequence and restriction map of this region is known (GenBank accession nos AC005803 and J03071), each of the contiguous fragments could be specifically probed. The data demonstrated that all regions tested were enriched in the loop fraction of the MAR preparation. This contrasted to the marked enrichment of Igμ intronic enhancer elements in the MAR fraction. Thus, the entire hGH LCR and hGH gene cluster was devoid of MAR determinants.

Sequences at HSI,II were introduced by a partial LINE-1 insertion in the primate lineage

In light of the detection of functional MARs by the MarFinder program in other systems (16), it was of interest to determine the origin of the strong false-positive MAR prediction in the case of the hGH LCR. One possibility was that this region represented a MAR segment duplicated from elsewhere in the genome that had subsequently lost function. To determine whether sequences at HSI,II resembled any other known MAR-containing regions, we performed a BLAST search of existing human genomic DNA sequence databases (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) with the 1.6 kb *Bgl*II fragment. Remarkably, this search resulted in 200 independent hits. The multiple genomic homology segments represented the unique L1PB2 subclass of LINE-1 elements. This subclass represents one of the earliest primate-specific LINE-1 elements. To delineate the relationship between this LINE and the putative MAR element, the HSI,II sequence was aligned directly to the deduced L1PB2 consensus sequence (26). This alignment revealed a highly conserved LINE 3′-UTR with an AT-rich terminus (Fig. 4A). Reanalysis of the HSI,II sequence with the MarFinder program using a narrower window (50 bp) localized the MAR potential peak precisely at the L1PB2 3′ A-rich tail (Fig. 4B). Removal of this AT-rich segment from the sequence resulted in the loss of the MAR potential peak. Thus, the *in silico* HSI,II-linked MAR potential reflected the recent insertion of a 5′-truncated L1PB2 LINE element 5′ to the hGH gene cluster, and did not correlate with nuclear matrix association in a cell-based assay.

DISCUSSION

We have previously described a LCR located between 15 and 32 kb upstream of the hGH-N gene. The major determinant of hGH LCR action in the pituitary, HSI,II, has been mapped to a site 15 kb 5′ to the hGH-N gene promoter. HSI,II is sufficient for the high-level, position-independent, somatotrope-specific, and developmentally appropriate activation of the hGH-N transgene (18,20,21). In the present study, we sought to determine whether the LCR-mediated long-range gene activation might be correlated with the presence of a MAR determinant(s) in this region. Using an algorithm based on the association of a set of putative sequence rules with known MARs, the region immediately encompassing HSI,II was predicted to contain MAR function (Fig. 1). However, MAR assays of nuclei from HeLa cells and from primary pituitary cells failed to confirm MAR elements associated with HSI,II (Fig. 2) or with any other regions in the LCR or hGH gene cluster (Fig. 3).

The role of MARs in the topological organization of the genome has suggested that they might have a role in transcriptional regulation. An emerging concept is that MARs organize the genome into discrete, autonomous regulatory domains. Studies in a number of systems support this model. The *Drosophila melanogaster* ftz, Sgs-4 (27), chicken lysozyme (28), and human β-globin genes (29) are each flanked by MARs required for the insulation of transgenes from position effects (22,30). The prevalence of topoisomerase II sites at known MARs (4,31) and evidence linking active chromatin and torsional stress in higher eukaryotes (32–34) suggests a mechanistic basis for such MAR-mediated functions. A number of MARs are also found in the proximity of transcriptional enhancers and may contribute to their ability to act over long distances. This enhancer facilitation may reflect sequestration of chromatin domains into specific subnuclear microenvironments and/or the regional modulation of chromatin accessibility. The most detailed evidence for the interaction of MARs with LCR elements and enhancers comes from studies

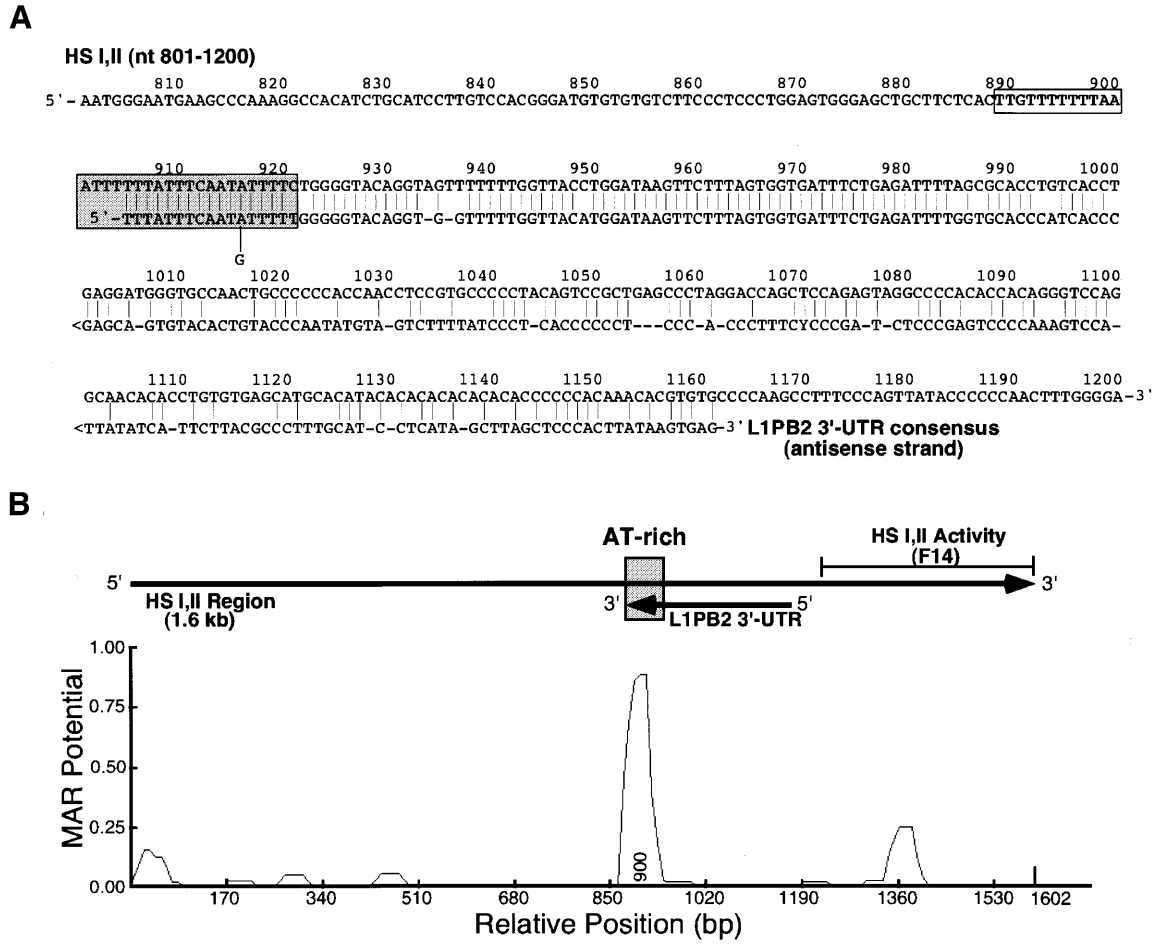

Figure 4. The *in silico* MAR potential was conferred by the 3′-UTR of a truncated LINE-1 element. (**A**) Alignment of the L1PB2 3′-UTR with HSI,II. Nucleotides 801–1200 of the 1602 bp HSI,II region (*Bgl*II restriction fragment; Fig. 1) are shown. The A+T-rich sequence that resulted in the significant *in silico* MAR potential at position 900, corresponding to the L1PB2 short 3′ A-rich tail, is indicated by a shaded box. The represented consensus sequence does not include the entire A-rich tail beyond the 3′-UTR because of its variable length. (**B**) The peak of *in silico* MAR potential coincides with the L1PB2 A-rich tail. The position of the peak MAR potential was further resolved by the MarFinder program using a 50 bp window.

of the intragenic mouse Igμ heavy chain LCR. The major enhancer element in this LCR is flanked by MARs (4,23). While these MARs have no appreciable effect on enhancer action in cell transfection studies (9,35–37), they have been found to markedly augment Igμ enhancer action in transgenic mouse B-cells. They extend the distance over which the Igμ enhancer can influence chromatin accessibility, core histone acetylation, DNA demethylation, and transcription (9,11,12).

In contrast to the multiple studies documenting activity of MARs in gene expression, a number of studies indicate that these effects may not be universal (e.g. 38, reviewed in 39). For example, site-directed mutations of MARs flanking the murine β-globin gene (40), the human Aγ-globin gene (41,42) and the endogenous murine Igμ LCR (43) do not appear to alter expression of the associated promoters. Thus, the role of MARs may be specific to certain genes or particularly evident in specific experimental contexts.

The lack of MARs within the hGH LCR suggests that HSI,II activates the hGH-N transgene over an intervening distance of 15 kb by a MAR-independent mechanism. It has been established that this region of the LCR is a target for histone acetylation in a somatotrope nucleus (25), possibly through recruitment of histone acetyltrasferase complexes by the POU-homeodomain factor Pit-1 (21,44). This recruitment triggers the modification of a 32 kb domain of chromatin acetylation extending 5′ to the most distal LCR determinant (HSV; –32 kb) and 3′ to the hGH-N promoter. This modification, targeted to HSI,II and subsequently spread throughout an extensive domain, may be sufficient to mediate hGH-N gene activation. Thus, the hGH LCR may utilize a mechanism of long-range gene activation that obviates the need for MAR function at this locus.

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