Recruitment of Heterogeneous Nuclear Ribonucleoprotein A1 in Vivo to the LMP/TAP Region of the Major Histocompatibility Complex*

Sequences containing the matrix recognition signature were identified adjacent to the LMP/TAP gene cluster in the human and mouse major histocompatibility complex class II region. These sequences were shown to function as nuclear matrix attachment regions (MARs). Three of the five human MARs and the single mouse MAR recruit heterogeneous nuclear ribonucleoprotein A1 (hnRNP-A1) in vivo during transcriptional up-regulation of the major histocompatibility complex class II genes. The timing of this recruitment correlates with a rise in mature TAP1 mRNA. Two of the human MARs bind hnRNP-A1 in vitro directly within a 35-bp sequence that shows over 90% similarity to certain Alu repeat sequences. This study shows that MARs recruit and bind hnRNP-A1 upon transcriptional up-regulation.

Matrix attachment regions (MARs) are short DNA sequences that bind to the ribonucleoprotein network that is generally referred to as the nuclear matrix or scaffold (1). A subset of MARs is believed to mediate the organization of chromatin into a higher order structure consisting of multiple topologically constrained loops attached at their bases to the matrix (2). A role for MARs in DNA replication has been proposed because many MARs contain origins of replication, and DNA unwindig, AT tracts, and DNase I-hypersensitive sites (1, 6). Binding of MARs to the matrix has not been assigned to unique DNA sequences but rather to sequences dispersed over several hundred base pairs. Recently, a unique bipartite sequence element was found in a large proportion of MARs, called the “MAR/SAR recognition signature” (MRS) (7). The MRS was reported to predict correctly the locations of MARs/SARs in several species from their genomic sequence alone.

In this study, we examined the role of MARs adjacent to the LMP/TAP gene cluster in the class II region of the human MHC. This genomic region was chosen as a model for both its sequence, transcription, and function. MARs are thought to position genes within the domain from adjacent regulatory elements (5).

MARs are identified biochemically by their selective retention in nuclear matrix preparations, which are derived from nuclei depleted of histones and most of the DNA. A set of characteristic motifs has been associated with MARs, including DNA unwinding elements, AT tracts, and DNase I-hypersensitive sites (1, 6). Binding of MARs to the matrix has not been assigned to unique DNA sequences but rather to sequences dispersed over several hundred base pairs. Recently, a unique bipartite sequence element was found in a large proportion of MARs, called the “MAR/SAR recognition signature” (MRS) (7). The MRS was reported to predict correctly the positions of MARs/SARs in several species from their genomic sequence alone.

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class II gene expression, PGE2 was added into the culture medium for 10% fetal calf serum at 37°C for 20 h as described previously (16).

PENDENT or loop (L) DNA fractions from MRC5 cells, IFN-γ up-regulated MRC5 cells, and NIH3T3 cells were grown and treated with IFN-γ (15). In some experiments mouse embryo monolayer NIH3T3 cells were used. These were grown and treated with IFN-γ as for MRC5 cells; however, E4 medium was used instead of RPMI 1640.

The B-lymphoblastoid cell line AHB, which constitutively expresses the classical MHC and the LMP/TAP genes at high levels, was grown in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in a 5% CO2 atmosphere. In experiments with up-regulated MRC5 cells, 200 units/ml IFN-γ (recombinant human IFN-γ, R & D Systems, Oxon, UK) was added to the medium for 24 h (15). In some experiments mouse embryonal monolayer NIH3T3 cells were used. These were grown and treated with IFN-γ as for MRC5 cells; however, E4 medium was used instead of RPMI 1640.

The lymphohistiocytic cell line AHB, which constitutively expresses the classical MHC and the LMP/TAP genes at high levels, was grown in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in a 5% CO2 atmosphere. In experiments to inhibit MHC class II gene expression, PGE2 was added into the culture medium for 20 h as described previously (16).

PCR Assay—The nuclear matrix-attached (M) and the matrix-independent or loop (L) DNA fractions from MRC5 cells, IFN-γ up-regulated MRC5 cells, AHB and NIH3T3 cells were isolated as described previously (17). Extractions with 25 mM LiCl, 0.65 M ammonium sulfate, or 2 M NaCl were employed in these separations. A PCR assay was used to identify specific DNA sequences in M- and L-fraction chromatin (18). The M- and L-DNA fractions, 50 ng each as determined by spectrophotometric measurement, were used as templates for amplification with corresponding PCR primer pairs (Table I). In all assays, the quantity of PCR products was maintained within the linear range (increasing the concentration of template or the number of cycles proportionally increased the signal). Aliquots of the PCR products were then electrophoresed in a 1.5% agarose gel, stained with ethidium bromide, and quantified densitometrically using Labworks 3.0 software. A control ratio between the sum of intensities of the M- plus L-fractions and the band intensity of PCR product using total genomic DNA as an internal control with the same primer pair as used in the M- and L-fractions was estimated. This ratio was always ~1 ± 0.021, confirming the equivalent efficiency of the PCR in the M- and L-fractions and in total genomic DNA. All PCR experiments were carried out in triplicate for three independent M- and L-fraction separations. Similar results were obtained and summarized in histograms.

Nuclear Extract Preparation—Nuclear extracts from MRC5 cells, IFN-γ up-regulated MRC5 cells, and AHB cells were prepared. The cells were collected and washed in Buffer A (146 mM sucrose, 100 mM KCl, 10 mM Tris, pH 7.0, 1.5 mM MgCl2, 1 mM DTT, 50 mM NaCl, 0.1% sodium deoxycholate, 0.5% Nonidet P-40, and 200 mM glycerol, 20 mM HEPES, pH 7.9, 1 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, and 50 mM NaCl). The reaction mixture was sonicated salmon sperm DNA, protease inhibitor (Sigma). The reaction mixture was preincubated at 24,000 × g for 20 min at 4°C. The extracts were aliquoted and stored at −80°C.

Pull-down of Proteins and Mass Spectrometry Identification—The pull-down of proteins specifically binding the MARs of interest was carried out as described previously (19) with slight modifications. The MARs were amplified from total genomic DNA by PCR using sets of specific primers (Table I) where the reverse primers were previously biotinylated by Biotin-Chem-Link kit (Roche Diagnostics). Approximately 80 pmol of each MAR was immobilized onto 400 µg of streptavidin magnetic particles (Roche Diagnostics) following the protocol supplied by the manufacturer. The pull-down was performed in a final volume of 550 µl containing 100 µg of nuclear protein extract, 10% glycerol, 20 mM HEPES, pH 7.9, 1 mM MgCl2, 1 mM DTT, 50 mM NaCl, 0.1 µg/µl poly(dI-dC) (Roche Diagnostics), and 1 µg/µl sonicated salmon sperm DNA, protease inhibitor (Sigma). The reaction mixture was preincubated for 10 min at room temperature; 400 µg of streptavidin magnetic particles with attached MARs were added and the mixture incubated for a further 25 min on a rotator. After magnetic separation,

| Table I | Primers, annealing temperature, and expected PCR products for amplified probes |
|---------|------------------|
| Sequence name | Primers | T annealing | Product length |
| MAR1 | 5'-CCCTGTTGTTTGTTGCTACA-3' (F) | 52 | 276 |
|  | 5'-GCCGAGACTCGTCTCTCAG-3' (R) | 167 |
| MAR2 | 5'-CACATCGACATATGGCACAAGA-3' (F) | 52 | 144 |
|  | 5'-GACTCTTCAAGCGTCTCGA-3' (R) | 310 |
| MAR3 | 5'-ACACCCCTGATATCCAAAGA-3' (F) | 52 | 144 |
|  | 5'-TCTTGGGGTAGGTGATAGAAG-3' (R) | 152 |
| MAR4 | 5'-CAATGCTGTTCTGCTTAC-3' (F) | 54 | 464 |
|  | 5'-GCTGACGAGCTGGCTTCAC-3' (R) | 267 |
| Human | 5'-AAAGGGTGGTGGAGCTCTT-3' (F) | 54 | 108 |
| Non-MAR1 | 5'-GCTGACGAGCTGGCTTCAC-3' (R) | 267 |
| Human | 5'-GCTCTCCCGCTACTTGAG-3' (F) | 54 | 416 |
| Non-MAR2 | 5'-CCATGCGACAGTTGAGTACG-3' (F) | 54 | 201 |
| Mouse MAR1 | 5'-GGTCTCCCCTTTGCTGATAC-3' (F) | 54 | 71 |
|  | 5'-CAGATGACGATTGCTGTCCT-3' (R) | 50 | 35 |
| Mouse | 5'-GACCTGTCACCTGGGACAC-3' (F) | 54 | 61 |
| Non-MAR1 | 5'-TGAGGAAACGCATCCATTGCAG-3' (R) | 50 | 35 |
| Mouse | 5'-AACACTTGGGAACCTGGTGCT-3' (F) | 54 | 201 |
| Non-MAR2 | 5'-GATCCACGGGACACAGGCGATG-3' (R) | 50 | 35 |
| Probe 1 | 5'-GCTGACGAGCTGGCTTCAC-3' (F) | 53 | 71 |
|  | 5'-CCTGACGAGCTGGCTTCAC-3' (R) | 50 | 35 |
| Probe 2 | 5'-CCATGCGACAGTTGAGTACG-3' (F) | 54 | 201 |
|  | 5'-GGTCTCCCCTTTGCTGATAC-3' (R) | 50 | 35 |
| Probe 3 | 5'-GGTCTCCCCTTTGCTGATAC-3' (F) | 54 | 201 |
|  | 5'-CAGATGACGATTGCTGTCCT-3' (R) | 50 | 35 |
| Probe 4 | 5'-CCATGCGACAGTTGAGTACG-3' (F) | 54 | 201 |
|  | 5'-GGTCTCCCCTTTGCTGATAC-3' (R) | 50 | 35 |
| Probe 5 | 5'-CCATGCGACAGTTGAGTACG-3' (F) | 54 | 201 |
|  | 5'-GGTCTCCCCTTTGCTGATAC-3' (R) | 50 | 35 |

* F indicates forward primer, and R indicates reverse primer.
were resuspended in 30 °C washing buffer included 0.1 mM KCl, 20 mM HEPES, pH 7.9, 1 mM MgCl2, 0.5 mM DTT). The first l of Laemmli sample buffer containing 7 M urea, and the eluted proteins were separated in a 12% Tris glycine pre-cast gel (Invitrogen). The gels were either silver-stained (Silver Technology, Inc.) or Coomassie-stained, and proteins of interest were cut out and processed further for mass spectrometry as described previously (20).

**hnRNP-A1 and p21 Cloning and Expression**—By using specific primer pairs (Table II) for RT-PCR, hnRNP-A1 and the cdk inhibitor, p21, were amplified from their start to stop codons from cytoplasmic hnRNP-A1 and p21 mature mRNA

| Sequence name                          | Primers                                      | T annealing | Product length |
|----------------------------------------|----------------------------------------------|-------------|----------------|
| hnRNP-A1 cDNA for cloning              | 5‘-AGTGCAGACCGAGCCATGATGTA-3’ (F)            | 56          | 963            |
|                                        | 5‘-TTAAATCTTCTGCCACTCCATAGCCT-3’ (R)         |             |                |
| p21 cDNA for cloning                   | 5‘-AGTCGAGACCGAGCCATGATGTA-3’ (F)            | 53          | 495            |
|                                        | 5‘-TTAGGCCTCTTCTTGGTGAAGA-3’ (R)             |             |                |
| hnRNP-A1 mature mRNA                   | 5‘-AAGCTTACGAGGAGGAGGAGCAGCAG-3’ (F)         | 56          | 279            |
|                                        | 5‘-GCAAAGGCGAGGAGGAGCAGCAG-3’ (R)            |             |                |
| rec-hnRNP-A1                           | 5‘-GATTGACGTACAGCAGCAG-3’ (F) (TT primer)    | 42          | 232            |
|                                        | 5‘-ATCAGAAGGGCTGTTATGTA-3’ (R)               |             |                |
| TAP1 mature mRNA                       | 5‘-GGATTCGTATGATGATGATGATGTA-3’ (F)          | 56          | 796            |
|                                        | 5‘-CTTGAGGAGGGTATGATGATGATGTA-3’ (R)         |             |                |
| LMP2 mature mRNA                       | 5‘-ACACAGGACGAGGACGAGGACG-3’ (F)             | 55          | 170            |
|                                        | 5‘-ATGGAGGACGAGGACGAGGACG-3’ (R)             |             |                |
| Mouse Lmp2 mature                      | 5‘-TTGTGGAGGCTCTTCTCCAG-3’ (F)               | 54          | 111            |
|                                       | 5‘-ATCGCTGGAGGCTCTTCTCAG-3’ (R)              |             |                |
| Mouse Tap1 mature                      | 5‘-GGGATGACTTCTGCTTCTGTGTG-3’ (F)            | 54          | 264            |
|                                       | 5‘-GGGACGACTCTGCTTCTGTGTG-3’ (R)             |             |                |

* F indicates forward primer, and R indicates reverse primer.

**Table II**

**Table III**

| Sequence name | Primers | Primer concentrations | Product |
|---------------|---------|-----------------------|---------|
| β-Actin       | 5‘-GGATGACGAGGAGGAGGAGGACG-3’ (F) | 90/500 bp | 115 bp |
| LMP2          | 5‘-GAAAAACGAGGACGAGGACG-3’ (R)   | 56/500 bp | 117 bp |
| TAP1          | 5‘-GCAAAGGCGAGGAGGAGGAGCAGCAG-3’ (R) | 100/500 bp | 110 bp |
| Pre-LMP2      | 5‘-CCCTGCTCATCCATGCTCTTC-3’ (F) | 300/300  | 123 bp |
| Pre-TAP1      | 5‘-GGGACGATTACCCACCCACGGCATC-3’ (R) | 300/300  | 103 bp |
| Human MAR4    | 5‘-GAGAAGAAGGAGGAGGAGGAGGAC-3’ (R) | 900/300  | 103 bp |
| Mouse MAR1    | 5‘-GAGAAGAAGGAGGAGGAGGAGGAC-3’ (R) | 300/300  | 61 bp |

* F indicates forward primer, and R indicates reverse primer.

- **Chromatin Immunoprecipitation (CHIP) and MAR Sequence Detection**—The MRC5 cells were transfected transiently with rec-hnRNP-A1 as described above. After 48 h of growth in culture, half of the cells were treated with IFN-γ at 200 units/ml for a further 24 h to up-regulated MHC gene expression. IFN-γ-treated and -untreated cells were collected (~5 × 10^6 cells each) and fixed in tissue culture medium containing 1% formaldehyde for 10 min at room temperature. All further steps of this assay were as described previously (21). Chromatin sonication was performed to produce DNA fragments in the range of 400–700 bp (electrophoretically determined in 1.5% agarose). The immunoprecipitation was performed with Anti-Xpress™-horseradish peroxidase antibody supplier (Invitrogen). Signal detection was performed by membrane incubation in Chemiluminescence Luminal Reagent (Santa Cruz Biotechnology, Inc.).

- **Western Blot**—Nuclear protein extracts and nuclear matrix protein extracts from hnRNP-A1 transfected MRC5 cells were isolated at 24-h intervals up to 96 h. The proteins were separated in a 12% Tris glycine pre-cast gel (Invitrogen) and transferred onto a nitrocellulose membrane using an electroblopting apparatus XCell SureLock™ (Invitrogen) according to the manufacturer’s protocols. Further procedures for detecting the recombinant hnRNP-A1 containing Xpress tag followed instructions of the Anti-Xpress™-horseradish peroxidase antibody supplier (Invitrogen). Signal detection was performed by membrane incubation in Chemiluminescence Luminal Reagent (Santa Cruz Biotechnology, Inc.).

- **Sequence detection**—The PCR products were again maintained sequence detection. Primer pairs used for MAR sequence detection are given in Table I. The quantity of PCR products was maintained within the linear range. Subsequently, aliquots of the PCR products were electrophoresed in a 1.5% agarose gel, stained with ethidium bromide, and quantified densitometrically using Labworks 3.0 software. The intensities of MAR fragments amplified from co-immunopre-
cipitated DNA were divided by the intensity of MAR fragments amplified from total genomic DNA (50 ng of DNA/reaction) using the same primer pairs. The calculated ratio (relative binding) indicates the enrichment of co-immunoprecipitated DNA in corresponding MAR sequences, compared with their number in the same amount of genomic DNA. All PCR experiments were carried out in quadruplicate for three independent CHIP analyses. Similar results were obtained and summarized in histograms. Control real time quantitative PCR experiments were performed for human MAR4 and the single mouse MAR1 as described below. Primers used in quantification experiments are given in Table III.

Detection of Pre-mRNAs and Mature mRNAs—Cytoplasmic RNAs isolated from different cell types (MRC5 and NIH3T3, MRC5 and NIH3T3 IFN-γ treated for 24 h, MRC5/rec-hnRNP-A1 transfected for 72 h, and MRC5/rec-hnRNP-A1 transfected for 72 h, and IFN-γ treated for 24 h, and AHB and AHB cells PGE 2-treated for 20 h) were used as starting templates for RT-PCR to monitor the levels of mature mRNAs. To assess the levels of pre-mRNAs, total nuclear RNAs isolated from the above human cell lines were examined. Cytoplasmic and total nuclear RNAs were isolated as described previously (22). The subsequent semi-quantitative RT-PCR was performed using specific primers shown in Table II and Qiagen® One-step RT-PCR kit (Qiagen) following the supplier’s recommendations. Control experiments with β-actin were carried out using standard primers (Promega). Aliquots of the RT-PCR products were separated in a 1.5% agarose gel, ethidium bromide-stained, and quantified densitometrically using Labworks 3.0 software. All RT-PCR experiments were carried out in triplicate for two independent RNA isolations. Similar results were obtained and summarized in histograms. Each relative expression value represents a ratio of the densities of specific mRNA transcripts to corresponding β-actin transcripts.

Real Time Quantitative PCR/RT-PCR—Cytoplasmic and nuclear RNAs were isolated from MRC5 cells and MRC5 transfected with p21 for 20 h and treated with IFN-γ for 0, 2, 4, 6, and 8 h. They were used as starting templates for real time quantitative RT-PCR. Aliquots of the RNAs were reverse-transcribed using random hexamers and MultiScribe reverse transcriptase according to the manufacturer’s instructions (Applied Biosystems). Primers were designed using Primer Express software (Applied Biosystems), taking into account intron/exon boundaries to ensure specific amplification of cDNA (Table III). Primers were designed to β-actin as an internal control for normalization of starting cDNA levels. Quantitative PCR was performed using SYBR Green PCR Master Mix according to the manufacturer’s instructions (Applied Biosystems), with the exception that 25-μl reaction volumes were used, with 45 cycles of amplification. Each of the primer pairs was optimized to ensure amplification of the specific product and absence of primer dimers (Table III). PCR was performed on the Taqman 7700 (Applied Biosystems). Following amplification, RT-PCR products were electrophoresed on agarose gels to check for the correctly sized product. The real time PCR results were analyzed using the sequence detection system software version 1.9 (Applied Biosystems). Gene expression levels were calculated using the comparative Ct method (ΔΔCt). ΔΔCt validation experiments showed similar amplification efficiency for all templates used (difference between line slopes for all templates less than 0.1). Expression levels of the genes were normalized to those in MRC5 cells without IFN-γ treatment. At least two independent experiments were performed for each gene. Similar results were obtained and summarized in histograms.

hnRNP-A1 recruitment in vivo to human MAR4 was examined by CHIP at 0, 2, 4, 6, 8, and 24 h after the start of IFN-γ treatment of MRC5 cells and p21-transfected MRC5 cells. Immunoprecipitated DNAs were used as templates in subsequent real time quantitative PCR experiments. Levels of protein recruitment were calculated according to the ΔΔCt method. Similar amplification efficiencies were shown for all templates used. Recruitment levels to MAR4 were normalized to that in MRC5 cells without IFN-γ treatment. Two independent experiments were carried out. Similar results were obtained and summarized in histograms. Recruitment of hnRNP-A1 to the single mouse MAR1 was examined in NIH3T3 cells 0 and 24 h after IFN-γ treatment. Results were analyzed as for the human MAR4.

AFM Scanning and EMSA—Direct hnRNP-A1 binding to the MARs was investigated by examining DNA-protein complexes by AFM topography imaging as described previously (23) and by EMISA. Sets of specific primer pairs were used for amplification of the probes for EMISA (Table I). Probes 1–5 were labeled with horseradish peroxidase using the NorthSouth Direct horseradish peroxidase labeling and detection kit (Pierce). Each probe (80 ng) was incubated with 100, 200, or 300 ng of hnRNP-A1 for 50 min in binding buffer (20 mM Tris-HCl, pH 7.4, 0.1 mM MgCl2).
of MHC class II gene expression were used to determine whether transcriptional status in the region is relevant to the functioning of these MAR sequences. The human fibroblast cell line MRC5 expresses the LMP2/TAP genes at low levels and does not express the classical MHC class II genes. Expression of all these genes is greatly increased when MRC5 cells are treated with IFN-γ for 24 h (25). The B-lymphoblastoid cell line AHB constitutively expresses these genes at high levels.

We found that all five genomic fragments containing the MRS from the LMP2/TAP gene region bound to the nuclear matrix, regardless of the extraction method used (Fig. 2). These fragments were thus designated MAR1–5. In all three cell types extracted with lithium 3,5-diiodosalicylate (LIS) and ammonium sulfate, MARs-1, -3, -4, and -5 were detected in nuclear matrix fraction only (R = 100%). In contrast, MAR2 showed substantially different levels of matrix binding in cells with different levels of MHC gene expression. Around 40% of MAR2 sequences were found in the nuclear matrix fraction isolated from MRC5 cells when ammonium sulfate was used, and 60%
when LIS was used. However, all the MAR2 sequences were bound to the nuclear matrices isolated from IFN-γ-treated MRC5 and AHB cells by both extraction procedures. In NaCl-extracted cells, a lower proportion of MARs 1–5 sequences (45–65%) bound to the nuclear matrix than in LIS and ammonium sulfate-extracted cells, and no significant differences were found in cells with different levels of MHC gene expression. Neither of the control sequences bound to the nuclear matrix in these cell types using the three extraction procedures.

MARs in the Human LMP/TAP Region Recruit hnRNP-A1 in Vivo—The five MARs from the LMP/TAP gene region were 3'-biotinylated and attached to streptavidin-coated magnetic particles. They were then used to pull-down proteins using nuclear extracts from cells with different expression levels of the adjacent genes. SDS-PAGE analysis showed that each MAR bound multiple proteins (Fig. 3A). However, one protein between 31 and 36 kDa showed substantial quantitative differences in these cells. Far more of this protein bound to MARs using nuclear extracts from IFN-γ up-regulated MRC5 cells and AHB cells, which have higher expression levels of the adjacent LMP2 and TAP1 genes and the classical MHC class II genes, than untreated MRC5 cells. This protein was identified by mass spectroscopy as the heterogeneous nuclear ribonucleoprotein A1 (hnRNP-A1) (Fig. 3B). Subsequent investigations thus focused on this protein, which is involved in mRNA processing and export from the nucleus.

By using specific primers for RT-PCR (Table II), the cDNA for hnRNP-A1 was cloned in full in the pcDNA4/HisMax-TOPO expression vector. This construct was then used to transiently transfect MRC5 cells. Western blot detection of the recombinant protein in nuclear protein extracts and nuclear matrix protein extracts isolated from the transfected cells revealed maximum expression and incorporation into the nuclear matrix 72 h after transfection (Fig. 4). In all subsequent experiments employing recombinant hnRNP-A1, protein binding to MARs and LMP/TAP gene expression was examined 72 h after transfection.

In vivo binding of hnRNP-A1 to MARs 1–5 in MRC5, IFN-γ-treated MRC5, and AHB cells was then compared by CHIP. Live cells were fixed with formaldehyde, DNA-hnRNP-A1 complexes immunoprecipitated, and binding analyzed by semi-quantitative PCR (Fig. 5). Substantially more hnRNP-A1 bound to MARs-1, -2, and -4 in IFN-γ-treated MRC5 cells (about 4-, 27-, and 10-fold, respectively) and in AHB cells (6-, 31-, and 12-fold, respectively) than in untreated MRC5 cells (Fig. 5, A and B). No differences were observed for MAR3 and MAR5. These experiments demonstrate significantly increased binding of hnRNP-A1 to MARs-1, -2, and -4 in cells where MHC class II expression, including the LMP/TAP genes, is up-regulated. Control experiments for the background signal as a result of nonspecific binding to antibody and protein A-Sepharose were performed by replacing anti-hnRNP-A1 with normal IgG. The background for each MAR detected by semi-quantitative PCR was less than 6% of the intensity of corresponding bands obtained for MRC5 cells using anti-hnRNP-A1 antibody (Fig. 5A). Real time quantitative PCR for recruitment of this protein to MAR4 was performed to confirm the accuracy of the semi-quantitative experiments (Fig. 5B, striped bars). Around 11.6-fold higher binding of hnRNP-A1 to MAR4 was detected in IFN-γ-treated MRC5 cells compared with untreated cells.
result correlates well with that obtained by semi-quantitative PCR for MAR4 (around 10-fold).

CHIP analysis was then performed on AHB cells following down-regulation of MHC class II gene expression, including LMP and TAP genes, by prostaglandin E2 (PGE2) (16) (Figs. 5, C and D, and 6). Substantially less hnRNP-A1 bound to

Fig. 5. CHIP analysis of in vivo recruitment of hnRNP-A1 to human MARs 1–5. A, recruitment of endogenous hnRNP-A1 to MARs 1–5 in MRC5 cells, IFN-γ up-regulated MRC5 cells, and AHB cells. 1.5% agarose electrophoresis of semi-quantitative PCR products using primer pairs specific for the five human MARs and goat anti-hnRNP-A1 antibody co-immunoprecipitated DNA fractions from MRC5, IFN-γ up-regulated MRC5, and AHB cells as templates. Amplification of the MARs from genomic DNA was carried out as internal controls. CHIP using MRC5 cells and normal goat IgG instead of specific antibody was performed as a background control. B, histograms of relative binding of endogenous hnRNP-A1 to MARs 1–5. Values show means ± S.E. for three independent CHIP experiments each analyzed in quadruplicate. Black bars, which represent results from the semi-quantitative PCR, are related to the left-hand side scale. Striped bars show hnRNP-A1 recruitment to MAR4 measured by real-time quantitative PCR (qPCR, see the right-hand side scale). C, recruitment of endogenous hnRNP-A1 to MARs 1–5 in AHB cells and PGE2 down-regulated AHB cells. Similar internal and background controls were used as in A. D, histograms of relative binding of endogenous hnRNP-A1 to the MARs. Values show the means ± S.E. for three independent CHIP experiments each analyzed in quadruplicate. E, recruitment of rec-hnRNP-A1 to MARs 1–5 in transiently transfected MRC5 cells and MRC5-transfected and IFN-γ up-regulated. CHIP with anti-rec-hnRNP-A1 antibody was performed on untransfected MRC5 cells as a background control. Amplification of the MARs from genomic DNA was carried out as an internal control. F, the values of relative rec-hnRNP-A1 recruitment to the MARs are shown as means ± S.E. for three independent CHIP experiments, each analyzed in quadruplicate.
MARs-1, -2, and -4 in these cells (4-, 8-, and 11-fold, respectively) than in untreated AHB cells, whereas binding to MARs-3 and -5 was unaffected. These findings confirm the correlation between MHC class II gene expression and the level of hnRNP-A1 binding to MARs-1, -2, and -4. Control experiments for the CHIP background were carried out as described for Fig. 5A, using AHB cells. The intensity of the control signal was less than 7% of the intensity of corresponding bands for PGE2-treated AHB cells using anti-hnRNP-A1 antibody.

In addition to up-regulating MHC class II genes, IFN-γ/H9253 treatment of MRC5 cells led to higher expression of hnRNP-A1 (Fig. 6). To test whether increased binding of hnRNP-A1 to MARs-1, -2, and -4 is a result of the generally increased hnRNP-A1 concentration in the nucleus following transcriptional up-regulation or whether this is a specific recruitment, CHIP was performed against recombinant hnRNP-A1 transfected into MRC5 cells. IFN-γ up-regulation of transfected cells did not affect the expression level of rec-hnRNP-A1 (Fig. 6). Substantially more of this protein bound to MARs-1, -2, and -4 in IFN-γ-treated MRC5 cells (about 3-, 24-, and 8-fold, respectively) compared with untreated cells (Fig. 5, E and F), suggesting that the increased binding is not simply due to the increased concentration of hnRNP-A1 in the nucleus. CHIP with anti-rec-hnRNP-A1 was carried out in untransfected MRC5 cells as a control. The intensity of control bands was less than 3% of the intensity of corresponding bands in transfected cells.

To investigate further the relationship between increased hnRNP-A1 binding to MARs-1, -2, and -4 and expression of the adjacent LMP2 and TAP1 genes, semi-quantitative RT-PCR was performed (Fig. 6A). Cytoplasmic RNAs isolated from MRC5 and IFN-γ-treated MRC5 cells were used as starting templates to quantitate mature LMP2 and TAP1 mRNAs, whereas nuclear RNAs were used to quantitate pre-mRNAs. The results are summarized in Fig. 6B. Higher levels of hnRNP-A1 from rec-hnRNP-A1 transfection led to 4–5-fold less LMP2 and TAP1 pre-mRNAs and higher levels of the corresponding mature mRNAs (Fig. 6). IFN-γ treatment resulted in a slight increase of LMP2 and TAP1 pre-mRNAs, whereas the
levels prior to pre-mRNA reflects the rapid activation of the splicing machinery and the coupled mRNA export (27), suggesting a relationship between hnRNP-A1 recruitment and mRNA processing.

hnRNP-A1 Binds Directly to Human MAR2 and MAR4 in Vitro—The protein pull-down and CHIP experiments showed that hnRNP-A1 bound to all five MARs in the human LMP2/TAP gene region (Figs. 3A and 5). To determine whether the protein binds directly to the MAR sequences, atomic force microscopy (AFM) scanning of MAR-hnRNP-A1 complexes was performed. In the AFM images shown in Fig. 5A, height is coded by color, with low regions depicted in dark brown and higher regions in increasingly lighter yellow tones. Only MAR2 and MAR4 showed direct binding of hnRNP-A1 (Fig. 5A). The biotinylated 3′-end of DNA appears as a small light spot (black arrows). The hnRNP-A1, shown as a lighter area over the DNA (white arrows), bound to a different position in MAR2 and MAR4. However, for each of these two MARs, the position of protein binding was consistent in all molecules examined. The hnRNP-A1-binding sites in DNA were measured using line profiles of 100 DNA-protein complexes for each MAR, and the results are summarized in Table IV. Experimentally measured DNA lengths are in good agreement with the theoretical range of lengths for A- or B-form conformations. These results showed that hnRNP-A1 binds MAR2 and MAR4 directly within the 35-bp sequence GGAGGATCGCYTGAGGCCAGGAGTTCAAGACCAGC (Y = T or C). This binding was confirmed by EMSA (Fig. 8, B and C), where hnRNP-A1 bound within probes 2 and 4 only, corresponding to the 35-bp sequence identified from AFM images. The 35-bp sequence is not present in MARs 1, 3, and 5. Further computational analysis showed this motif to be over 90% similar to the oldest types of Alu repeat sequences, AluJo and FLAM_C (Fig. 5D) and to contain a polymerase III promoter element (B box) and several hormone-response elements (HREs), which were shown previously to function as transcription factor binding sites (28).

Conservation of the MRS and hnRNP-A1 Recruitment—We identified a single MRS-containing sequence 5′ of the mouse Lmp/Tap genes (Fig. 9A). A PCR assay with L- and M-fractions as templates isolated after LIS extraction of mouse NIH3T3 fibroblast cells demonstrated that around 50% of the single mouse MAR1 sequences bound to the nuclear matrix in NIH3T3 cells and 60% in IFN-γ-treated NIH3T3 (Fig. 9B and C). This sequence thus functions as a real MAR. Control random sequences from the same gene region, which do not contain the MRS, did not show any binding to the nuclear matrix (Fig. 9B and C). These observations confirm that the MRS is a conserved property of MARs in different species (7) (Table V). RT-PCR analysis revealed that IFN-γ treatment increased expression of the Lmp2 and Tap1 mouse genes around 4–5-fold (Fig. 10, A and B).

In vivo binding of hnRNP-A1 to mouse MAR1 was compared using CHIP of soluble chromatin fragments from NIH3T3 and IFN-γ up-regulated NIH3T3 cells by anti-hnRNP-A1 antibody (reactive against both human and mouse hnRNP-A1). Control experiments for the CHIP background were carried out as described for Fig. 5; however, NIH3T3 cells were used in this assay. The background found was less than 8% of the intensity of corresponding bands for NIH3T3 cells obtained by anti-hnRNP-A1. Relative binding of hnRNP-A1 to the mouse MAR was assessed by semi-quantitative (black bars) and quantitative (striped bars) PCR (Fig. 10, C and D) as described for human MARs. Approximately 10–12-fold higher in vivo binding of the protein to mouse MAR1 was found in both PCR assays in IFN-γ-treated cells compared with that in untreated cells.
DISCUSSION

Regulation of gene expression in eukaryotes occurs at many levels, including interactions of DNA with transcriptional activators and repressors, DNA methylation, histone modifications, and alternative splicing of mRNA (29). By using the MHC as a model, we previously demonstrated rapid alterations in large scale chromatin structure upon transcriptional activation with IFN-γ (30). This work examines interactions of non-coding DNA in the MHC with proteins in cells having different levels of MHC class II gene expression. We show substantial recruitment in vivo of a major mRNA processing protein hnRNP-A1 to certain MARs in the MHC during transcriptional activation of the MHC class II genes. Although the nuclear matrix is known to contain hnRNP-A1, this is the first evidence that MARs recruit this protein.

Our experiments showing the MRS contained within MARs

![Fig. 8. In vitro binding of hnRNP-A1. A, AFM topography images of human MARs 1–5 after incubation with hnRNP-A1 and fixation of the complexes onto mica. In the images, height is coded by color, with low regions depicted by dark brown and higher regions in increasingly lighter tones in yellow from 0 Å. Only MAR2 and MAR4 showed formation of complexes with hnRNP-A1 in vitro. The black and white arrows mark the positions of 3’-end biotinylation and protein binding, respectively. B, scheme of the probes amplified for EMSA by PCR. Probes 2 and 4 (35 bp each) correspond to the 35-bp sequence for binding hnRNP-A1 identified from AFM experiments. C, probes 1–5 labeled with horseradish peroxidase (lanes a), probes incubated with 100, 200, or 300 ng of hnRNP-A1 in the presence of 0.1 μg/μl poly(dI-C) (lanes b, d, and f, respectively), probes incubated in the presence of 0.1 μg/μl poly(dI-C), and 1 μg/ml sonicated salmon sperm DNA (lanes c, e, and g, respectively) were separated in a 2% agarose gel. The DNA signal was detected by chemiluminescence. D, an alignment of the 35-bp hnRNP-A1 binding motif (hnRNP-A1B) with FLAMs and Alu left ends. Positions are numbered with respect to the FLAM_C sequence and matches to hnRNP-A1B shown in red or blue (to degenerate code). The B box consensus of the promoter for polymerase III is also shown with the invariant bases underlined. The four HREs are superimposed in purple.]

### Table IV

| MARs | Average length of DNA | Average length of DNA covered by hnRNP-A1 | Average length from 3’-end of DNA to the binding of protein |
|------|-----------------------|------------------------------------------|----------------------------------------------------------|
| MAR1 | 84.6 ± 5.7            | 14.8 ± 0.6                               | 19.1 ± 0.8                                               |
| MAR2 | 52.3 ± 3.4            | 14.8 ± 0.6                               | 19.1 ± 0.8                                               |
| MAR3 | 47.1 ± 2.9            | 14.8 ± 0.6                               | 19.1 ± 0.8                                               |
| MAR4 | 47.1 ± 2.9            | 15.1 ± 0.9                               | 70.3 ± 4.1                                               |
| MAR5 | 47.3 ± 3.5            | 15.1 ± 0.9                               | 70.3 ± 4.1                                               |
in the LMP/TAP genomic region of the human and mouse genomes confirms and extends the findings of van Drunen et al. (7) that the MRS is a conserved property of MARs, implying an essential function for these sequences. A further level of conservation is seen in the finding that these MARs recruit hnRNP-A1 during transcriptional up-regulation in human and mouse cells, indicating a conserved role for these MARs in mRNA processing.

The Nuclear Matrix—The nuclear matrix can be defined as the non-chromatin fibrogranular ribonucleoprotein network in the nucleus that is readily observed in unextracted cells using the electron microscope (31, 32). This network fills the nuclear interior and is connected to the nuclear lamina. Much debate has focused on whether the nuclear matrix is a structural and functional entity in itself, or whether it is induced during extraction procedures or cell fixation prior to electron microscopy examination (15, 33). It has been suggested recently that, irrespective of the nature of the matrix in vivo, certain elements of the isolated matrix may reflect a significant “chemical footprint” of protein-protein and protein-nucleic acid interactions occurring during nuclear metabolism (15). Our finding of recruitment in vivo of hnRNP-A1 to MAR sequences in the MHC during up-regulation of MHC gene expression supports the view that we have discovered meaningful interactions of MARs with hnRNP-A1, which is a major component of the nuclear matrix.

Human MARs 1–5 were retained in nuclear matrix fractions isolated by LIS, ammonium sulfate, or NaCl treatment, followed by nuclease digestion. No MAR sequences were found in the loop fraction in LIS- and ammonium sulfate-extracted preparations except MAR2 in MRC5 cells, but they were all found in the loop fraction in NaCl-extracted preparations. Therefore, NaCl extraction releases these MARs from the matrix fraction so that they are found in the loop fraction in a proportion of the cells. These data are in agreement with the recent report that 2 M NaCl extraction plus DNase I/RNase A digestion disrupts chromosome territory architecture, in contrast to extraction with ammonium sulfate at the same ionic strength (17, 34).

The difference between NaCl and ammonium sulfate extraction might be explained by the greater blocking of electrostatic interactions between DNA and nuclear matrix proteins by NaCl ions compared with that by ammonium sulfate. Na+ and Cl− are smaller than NH4+ and SO4 2−; therefore, they penetrate the DNA-protein complexes more effectively. In addition, NaCl ions are known to have a thicker solvation shield than ammonium sulfate ions. Extraction by NaCl thus releases the negatively charged DNA from the positively charged amino acid residues of the nuclear matrix proteins. Release of DNA from the nuclear matrix is also dependent on the use of RNase treatment (34). This is likely to be due to the tight attachment of MARs to small RNA species in the nuclear matrix as well as to proteins (35).

Recruitment of hnRNPA1—The hnRNP proteins are a major

| Accession number | Species | Start position | Motif 1 | Motif 2 | Gene |
|------------------|---------|----------------|---------|---------|------|
| X87344           | Human   | 93,872         | ataataagctgtacgta (−82n−) ataataa (−90n−) | LMP2 |
| U35323           | Mouse   | 47,645         | taataaaaaagc (−29n−) ataataa (−168n−) | Lmp2 |

Table V

An example for conservation of MRS pattern between human and mouse

Fig. 9. Scheme of the Lmp/Tap region of the mouse MHC. A, candidate MAR in the Lmp/Tap gene region containing the MRS pattern (black box 1). B, L- and M-DNA fractions were isolated from NIH3T3 and IFN-γ up-regulated NIH3T3 mouse cells by 25 mM LIS extraction. These fractions were used in the subsequent PCR assay as templates. Positive and negative internal controls were carried out with total genomic DNA and pBR322 templates, respectively, for each sequence. Two non-MAR sequences from the same gene region were tested for their nuclear matrix binding ability as a control for assay specificity. The PCR products were separated in a 1.5% agarose gel. C, R values for the sequences binding to the nuclear matrix were estimated as in Fig. 2B. Values show the means ± S.E. for three independent PCR assays.
component of the nuclear matrix, and of these, hnRNP-A1 is one of the most abundant (33). Expression of recombinant hnRNP-A1 and Western blot analysis of nuclear matrix extracts from MRC5 cells unambiguously showed that it is a nuclear matrix protein. Our experiments confirmed that hnRNP-A1 is a major protein binding to the MARs in the LMP/TAP region.

hnRNP-A1 is an essential protein for mRNA maturation and interacts with splicing factors to regulate alternative splicing (36). The protein shuttles continuously between the nucleus and cytoplasm and is also believed to function, together with other hnRNPs, as a carrier for mRNA during export to the cytoplasm (37). Accumulation of hnRNP-A1 in the nucleus is transcription-dependent and is blocked by RNA polymerase II inhibitors (38). Furthermore, it has been shown that this accumulation can be triggered in early mouse embryos by the appearance of nascent transcripts, to which it is proposed to bind (39). IFN-γ up-regulates the expression of over 200 genes (25), including hnRNP-A1 (Fig. 6). We would thus expect a substantially increased concentration of nuclear hnRNP-A1 in fibroblasts treated with IFN-γ and in AHB cells where all the MHC class II genes are expressed, compared with untreated fibroblasts.

Our experiments revealed that MARs-1, -2, and -4 recruit hnRNP-A1 and recombinant hnRNP-A1 in vivo during up-regulation of gene expression (Fig. 5) increasing its concentration in the vicinity of the LMP2 and TAP1 genes. However, pull-down experiments in vitro (Fig. 3) showed substantially more of this protein bound to all the MARs in up-regulated cells. This discrepancy could be explained by the ability of all MARs to bind hnRNP-A1 directly or through other proteins because CHIP background controls showed signal less than 6% of the intensity of corresponding bands for MRC5 cells (Fig. 5). Under in vitro conditions, concentration of a particular protein is likely to be the major factor for binding higher or lower amounts of it to a specific DNA sequence. However, under in vivo conditions IFN-γ treatment of MRC5 cells transiently transfected with hnRNP-A1 did not influence transcription of the recombinant gene (Fig. 6). This suggests that recruitment of hnRNP-A1 in vivo to the MARs is not simply due to an increase in the concentration of the protein but is more likely to be related to transcriptional up-regulation of the adjacent genes and the simultaneous requirement for mRNA processing machinery. Recently, it was found that hnRNP-A1 plays an important role in the negative regulation of splicing by binding to exonic silencer elements and counteracting the positive activity of SR proteins (40). The timing of recruitment of this protein to MARs (Fig. 7) suggests that they may serve to enhance splicing by sequestration of the protein. Nuclear speckles are likely to be a depot for hnRNP-A1, because they serve as transient storage and assembly sites for pre-mRNA splicing factors that associate with sites of active transcription (41, 42).

Although the role of hnRNP-A1 in mRNA processing and transport has been studied in detail (37), there are few reports of interactions of the protein with DNA. hnRNP-A1 has been shown to bind an ATTG sequence motif within the cell cycle regulatory unit in the human thymidine kinase gene promoter region, suggesting a role for the protein in regulating gene expression (43). hnRNP-A1 may also play a role in telomere biogenesis, because it simultaneously binds single-stranded telomeric DNA and telomerase RNA in vitro (44). In addition, deficiency of hnRNP-A1 in a mouse erythroleukemia cell line leads to short telomeres (45). This report is the first showing recruitment and binding of hnRNP-A1 to MARs. We extended our investigation to the mouse H2-mb1 gene region and found similar recruitment by one of two MARs theoretically predicted by the MRS and experimentally shown to function as MARs (results not shown). Further studies need to be performed to determine whether MARs in other regions of the MHC or other up-regulated genomic regions bind hnRNP-A1.

Identification of a Consensus Sequence for Direct Binding of hnRNP-A1—Although we showed that these MARs bound hnRNP-A1, it was not clear whether binding is due to direct interactions or through some other protein(s). Formaldehyde used for fixation in CHIP analysis is a tight (2 Å) cross-linking agent, efficiently producing both protein-nucleic acid and protein-protein cross-links in vitro (46). To examine MAR/hnRNP-A1 binding more closely, we studied the human MAR/hnRNP-A1 complexes in vitro using AFM topographic imaging (Fig. 8). Only MAR2 and MAR4 were found to bind hnRNP-A1 directly, suggesting that MAR2-1, -3, and -5 bind the protein indirectly through one or more other proteins possibly from the hnRNP family (47).
motif almost identical to this 35-bp motif was shown in vitro in double-stranded DNA from human chromosome band 11q13 to bind hnRNP-A1 directly as well (23). The genomic sequences on band 11q13 surrounding these motifs were identified as “potential MARs” using “MAR-Finder” (48) (data not shown). The 35-bp binding motif does not show any similarity to the hnRNP-A1-binding motif in the thymidine kinase gene promoter region (43) or to the 20-mer consensus sequence identified in RNA for high affinity binding to hnRNP-A1 (49).

Subsequent sequence comparisons showed that the 35-bp hnRNP-A1-binding motif maps to an ancient family of Alu repeat sequences (FLAM-C and AluJo). Alu repeats are known to recruit regulatory proteins (28, 50), and these particular forms of Alu are twice as abundant in the LMP/TAP region as compared with the genome average (51). Furthermore, analysis of the human genome sequence reveals preferential retention of Alu repeats in G + C-rich (gene-rich) regions, suggesting a role for these repeats in gene expression (52). The HREs in retinoic acid receptors provide further evidence that so-called “junk DNA” has biological function.

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