Role of DNA Methylation in Transcription of Human Endogenous Retrovirus in the Pathogenesis of Systemic Lupus Erythematosus

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ABSTRACT. ***Objective.*** We recently reported that transcription of human endogenous retrovirus (HERV) clone 4-1-like sequences is increased in patients with systemic lupus erythematosus (SLE). We therefore investigated the role of DNA methylation in the transcription of this HERV.

Methods. The effect of a demethylating agent, 5-aza-deoxycytidine (5-aza C), on the transcription of HERV clone 4-1 in healthy individuals and patients with SLE was examined using reverse transcriptase-PCR and real-time quantitative PCR.

Results. 5-aza C increased clone 4-1-like messenger RNA in healthy controls, but not in patients with SLE.

Conclusion. Defects of methylation may contribute to the transcription of HERV in patients with SLE and this may be related to the pathogenesis of SLE. (J Rheumatol 2002;29:1678–82)

Key Indexing Terms:
HUMAN ENDOGENOUS RETROVIRUS
TRANSCRIPTION
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METHYLATION

Retroviruses, including endogenous retroviruses, have been repeatedly suggested as etiological factors for autoimmune rheumatic diseases such as systemic lupus erythematosus (SLE) in mice and humans[1,2]. Human endogenous retrovirus (HERV) is inherited in a stable Mendelian fashion and makes up 0.1–0.6% of human DNA, thus contributing substantially to the architecture of the human genome[3]. HERV generally lacks the extracellular phase that is normally characteristic of retroviruses[4]. Reports have suggested that expression of HERV proteins induced by activation of HERV sequences may play an important role in the induction of autoimmune diseases such as SLE[5-9]. We have reported that the transcription of HERV clone 4-1 is increased and its mRNA is detectable in patients with SLE, but not in healthy individuals, by reverse transcriptase (RT)-polymerase chain reaction (PCR)[2,10-13], although we cannot completely rule out the possibility that the virus detected by our PCR primers was not actually clone 4-1 because there are many members of the HERV-E family that are genetically similar to this clone[4]. Despite this limitation, these phenomena indicate that HERV clone 4-1-like sequences may play an important role in the etiology of SLE in certain patients.

Cytosine methylation of the regulatory sequences of some genes is associated with transcriptional inactivation, while hypomethylation of these sequences is associated with active transcription[14]. Studies have detected hypomethylation (implied by low methyltransferase activity) in T cell nuclear proteins from patients with SLE and have shown that DNA methylation inhibitors such as 5-aza-deoxycytidine (5-aza C) induced autoreactivity and autoantibody production *in vivo*[15-17].

Based on these findings, we examined the effect of a demethylating agent, 5-aza C, on the transcription of HERV clone 4-1-like sequences in order to clarify its mechanism of transcription in SLE.

MATERIALS AND METHODS

Patients. The patients (5 women aged 23–45 yrs) tested in this study were diagnosed as having SLE according to the 1982 revised criteria of the American College of Rheumatology[18]. Control samples were obtained from age and sex matched healthy volunteers. The main clinical manifestation in the 5 SLE patients was nephropathy, and they showed high serum levels of

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anti-DNA antibodies and low CH50 levels. Three patients had not received any steroids and the other 2 patients had been taking prednisolone for between one and 24 months. No patient received any immunosuppressive agents before the collection of samples and none was pregnant.

Cell culture. Peripheral blood mononuclear cells (PBMC) were separated from blood samples of the SLE patients and controls by centrifugation on a Ficoll-Paque cushion. Culture was performed in a 5% CO_2 incubator at 37°C using RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Gibco Laboratories, Grand Island, NY, USA). Cells were cultured with adequate concentrations of concanavalin A (Con A; Sigma Chemical Co., St. Louis, MO, USA) or 5-aza C (Sigma) for 48 h.

PCR analysis. Total mRNA from PBMC (2×10^6) was isolated using a QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA, USA). The cDNA was obtained from purified RNA using a First Strand cDNA Synthesis Kit (Amersham International, Buckinghamshire, England) with avian myeloblastosis virus (AMV) RT. Briefly, mRNA (2 μg) was incubated with 2.5 μg of oligo-dT, 10 μl of the first strand synthesis reaction buffer, 2.5 μl of sodium pyrophosphate solution, 5 μl of dNTP mixture, and 5 μl of human placental ribonuclease inhibitor in the presence of AMV RT (20 units) at 42°C for 50 min.

The sequence of HERV clone 4-1 was described by Repaske, *et al*[19]. RT-PCR using primers for the HERV clone 4-1 *gag* region was performed with an Expand High Fidelity PCR System (Roche Molecular Systems, Pleasanton, CA, USA). The following primers were used for PCR: 5'-ATG GAT CAG ACA CAA AAT AAG CC-3' (*gag* primer 1; nt 1042–1064) and 5'-TCA GTC CAA TAA CCC TTC TGC-3' (*gag* primer 2; nt 2629–2649) (Figure 1). For PCR amplification, we used 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 90 s, and elongation at 72°C for 2 min. To examine contamination by proviral DNA, samples containing mRNA without RT were also amplified under the same conditions. The PCR products were run on 1% agarose gel containing 0.5 μg/ml ethidium bromide.

Quantitative analysis of clone 4-1-like mRNA. Quantitative analysis of HERV clone 4-1 *gag*-like mRNA was performed by real-time quantitative (RQ)-PCR using a TaqMan fluorogenic system (ABI Prism 7700 Sequence Detection System; Perkin Elmer, Foster City, CA, USA) as described[20,21]. This system employed the following primers: 5'-CAC ATG GTG GAG AGT CGT GTT T-3' (*gag* primer 3; nt 1693–1714) and 5'-GCT TGC GGC TTT TCA GTA TGT G-3' (*gag* primer 4; nt 1772–1793) (Figure 1). Both primers were derived from the sequence encoding p30*gag* of HERV clone 4-1. The TaqMan probe for the HERV clone 4-1 *gag* region was labeled with FAM reporter dye (6-carboxytetrafluorescein) at the 5' end and with TAMRA (6-carboxytetramethylrhodamine) at the 3' end as the quencher dye: 5'-TTC ACC TCT GCC GAC CTT CTC AAC TGG A-3' (probe; nt 1726–1756) (Figure 1). For each PCR, a master mix was prepared on ice with 10× TaqMan buffer, 5 mM $MgCl_2$, 200 μM dATP, dCTP and dGTP, 400 μM dUTP, 300 nM of each primer, 150 nM of the probe, and 1.25 units of AmpliTaq Gold DNA polymerase (TaqMan PCR core reagent kit; Perkin Elmer). Then 5 μl of each appropriately diluted RT sample was added to 20 μl of the PCR master mix. The amplification conditions comprised an initial denaturation step at 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 65°C for 1 min. The cDNA standard template containing the HERV clone 4-1 *gag* and *pol* cDNA sequences (from nt 1630 to nt 2893) was obtained by PCR and cloning using a pGEM-T-Vector (Promega Corp., Madison, WI, USA). The standard curve for reverse transcription was drawn from 4 data points (5×10^2, 5×10^3, 5×10^5, 5×10^6 copies). The stimulation index (SI) was calculated as mean amount of mRNA (standard deviation < 15%) obtained by 3 separate experiments in normal PBMC treated with 5-aza C divided by that from untreated normal PBMC.

Statistical analysis. Statistical analysis was performed using Student's t test.

RESULTS

Detection of clone 4-1 mRNA. As shown in Figure 2, a fragment of roughly 1600 bp of the clone 4-1 *gag*-like region was amplified by RT-PCR using primers 1 and 2 for all the SLE patients. Similar fragments were not detected in the absence of RT, so the possibility of contamination by clone 4-1 proviral DNA was ruled out (data not shown). This fragment was not detected in the controls (Figure 2). In contrast, RT-PCR analysis of 5-aza C-treated PBMC from controls showed clone 4-1 *gag*-like mRNA, although it was not detected in control cells cultured without 5-aza C. The effect of 5-aza C on this transcription was observed to be concentration dependent (lanes 1–3 in Figure 3). The mRNA was

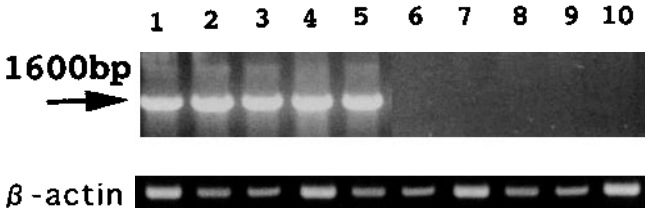

Figure 2. Detection of clone 4-1 mRNA in PBMC from SLE patients (lanes 1–5) and its absence in PBMC from controls (lanes 6–10).

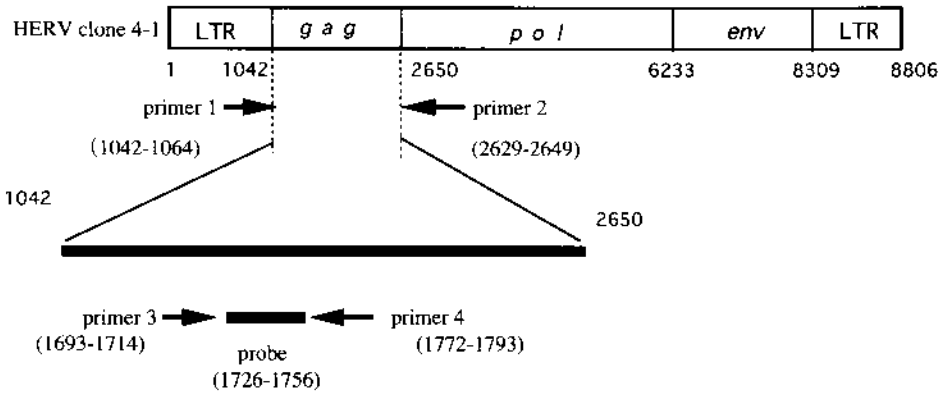

Figure 1. Structure of clone 4-1. Figures are nucleotide numbers.

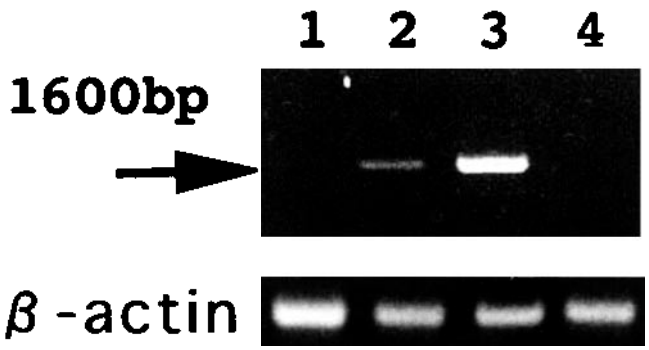

Figure 3. Detection of clone 4-1 mRNA in 5-aza C treated (lanes 1, 2, 3) or Con A treated (lane 4) PBMC from controls. Amount of 5 aza-C in lanes 1, 2, and 3 is 0, 50, 100 μM, respectively. Concentration of Con A in lane 4 is 10 μg/ml. Absolute amounts of mRNA measured by RQ-PCR in lanes 1–4 are as follows: lane 1, 72 copies/μg; lane 2, 381 copies/μg; lane 3, 869 copies/μg; lane 4, 70 copies/μg.

not detected in Con A stimulated normal PBMC (lane 4 in Figure 3).

Effect of 5-aza C on clone 4-1-like mRNA expression. Recently, a novel method for measuring mRNA has become available. This assay (the TaqMan assay, RQ-PCR) exploits the 5'–3' nuclease activity of Taq polymerase to detect and quantify specific PCR products, and the sensitivity of RQ-PCR is greater than that of RT-PCR[12,20,21]. Using this assay, we examined the effect of 5-aza C on the expression of clone 4-1 *gag*-like mRNA. As shown in Figure 4, the mRNA was significantly increased by 5-aza C treatment in 4 out of 5 PBMC samples from controls (controls 1, 2, 3, and 4). This effect was observed to depend on the 5-aza C concentration (Figure 4). However, 5-aza C treatment of PBMC from SLE patients had no perceptible influence on the expression of this mRNA (data not shown). In addition, Con A stimulation could not induce quantitative enhancement of clone 4-1-like mRNA in normal PBMC, as also shown by RT-PCR analysis (Figure 5).

DISCUSSION

HERV clone 4-1 is a member of the HERV-E family that shows homology with Molony murine leukemia virus (MuLV) and has a sequence roughly 8.8 kb long that includes open reading frames in the *gag*, *pol*, and *env* regions[19]. This HERV is widely found in human genomic DNA, and recent studies using human sequence data banks have shown that there are about 85 copies of clone 4-1 at 7

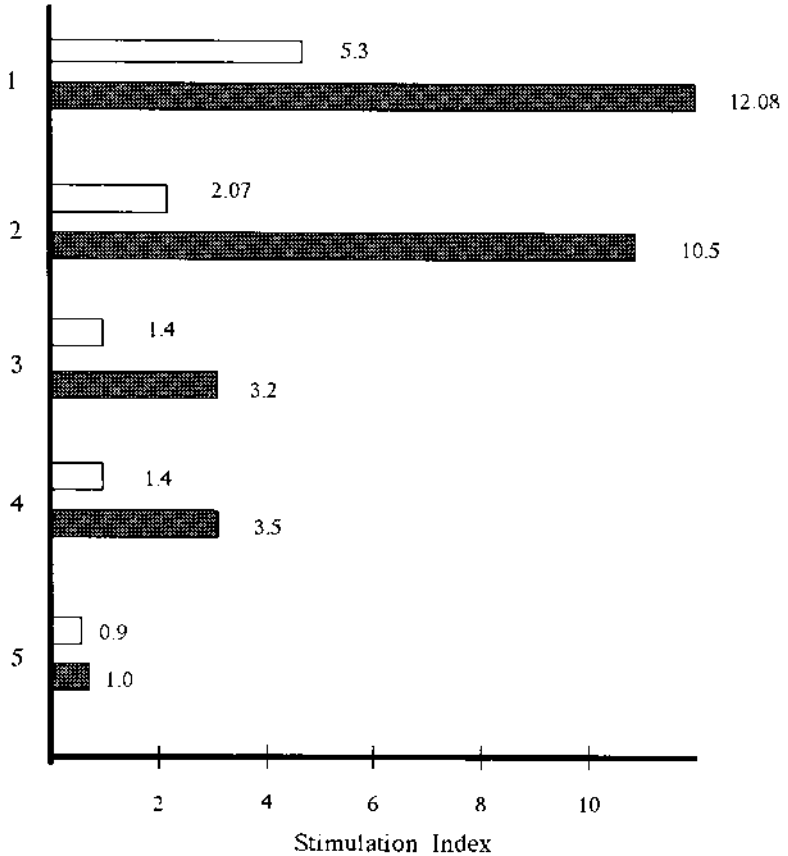

Figure 4. Effect of 5-aza C on the quantitative expression of clone 4-1 mRNA in PBMC from controls. Results are presented as the stimulation index (SI). Concentrations of 5 aza-C: white bars, 50 μM; black bars, 100 μM. There was a significant difference of SI between PBMC cultured with and that without 5 aza-C (100 μM) in all 5 healthy controls ($p < 0.001$).

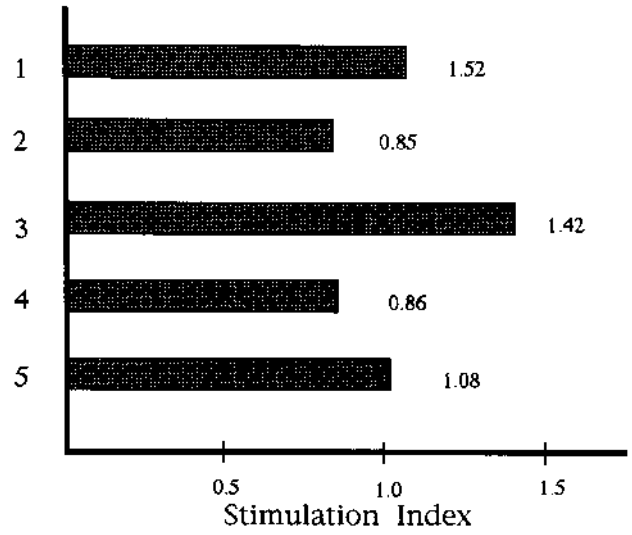

Figure 5. Effects of Con A on the quantitative expression of clone 4-1 mRNA by PBMC from 5 controls. See legend to Figure 4. There was no significant difference of SI between PBMC cultured with or without Con A (10 μg/ml) in all 5 controls. Absolute amounts of mRNA measured by RQ-PCR without 5-aza C in Figures 4 and 5 (sample numbers 1–5) are as follows: culture 1, 72 copies/μg; 2, 102 copies/μg; 3, 94 copies/μg; 4, 165 copies/μg; 5, 82 copies/μg.

to 11 integration sites in human chromosomes[22-24]. Recently, we reported that HERV clone 4-1-like sequences were strongly transcribed in SLE patients compared to healthy controls (the average amount of clone 4-1-like mRNA in 15 patients and 15 controls was 3173 ± 692 and 31 ± 12 copies/μg, respectively)[12], and we also found antigens to this HERV and serum antibodies in some SLE patients, but not controls[2,10-13,25]. Further, steroid treatment dramatically reduced the amount of clone 4-1-like mRNA in SLE patients, with steroid mediated improvement of SLE disease activity in each patient, although there is no direct relationship between the amounts of mRNA and SLE related clinical symptoms or disease activity as far as we know from our previous results[11,12].

The loss of competence to replicate viral proteins by HERV is generally thought to be due to the presence of several interrupters, including termination codons, deletions, frame shift mutations, or methylation sites. Our results indicate that DNA methylation may contribute to the lower transcription of HERV clone 4-1-like sequences in normal individuals because a demethylating agent, 5-aza C, can facilitate the sequence transcription even in healthy people (Figures 3 and 4), as well as transcription of other HERV[26]. Conversely, it is possible that methylation of HERV genes is impaired and that transcription occurs in patients with SLE because 5-aza C cannot increase their quantitative expression of clone 4-1-like mRNA, although further investigations of the effect of 5-aza C on the expression of other genes than clone 4-1 as controls are required to clarify this. Supporting this concept, previous studies revealed hypomethylation in SLE T cells, and suggested that this may be related to autoimmunity in SLE[15]. Further, intravenous injection of 5-aza C treated CD4+ T cells or T helper (Th)-2 cells into mice is reported to induce a lupus-like disease. This phenomenon has been attributed to enhancement of the effect of 5-aza C on the induction of autoreactivity in CD4+ T cells, and an overexpression of leukocyte functional associated antigen (LFA)-1 on cells induced by 5-aza C is reported to contribute to such autoimmunity[16,17]. In addition, such 5-aza C mediated autoimmune phenomena may be related to the transcription of endogenous retroviruses and the subsequent production of viral components that are important for the pathogenesis of model SLE in mice[27].

On the other hand, we found that Con A stimulation failed to induce transcription of clone 4-1-like sequences (Figures 3 and 5). This indicates that T cell activation does not result in increased transcription of the sequences, although SLE lymphocytes are generally activated *in vivo*[28], and that the mechanisms of mitogen related cell activation and 5-aza C induced demethylation involve different pathways of gene transcription. Using RQ-PCR (which is more sensitive than RT-PCR), clone 4-1-like mRNA is detectable even in the controls, although the quantitative level is extremely low compared to patients with SLE[12,13] (the average amount of mRNA from 2×10^6 PBMC incubated without 5-aza C in 5 SLE patients and 5 controls in this study was 1636.2 ± 457 and 82.2 ± 51 copies/μg, respectively). Even after 5-aza C treatment, the amount of clone 4-1-like mRNA was lower in normal lymphocytes than in cells from SLE patients (Figure 3), so we cannot exclude the possibility of factors other than methylation, such as enhancer activity in the long terminal repeat area, also promoting the transcription of SLE clone 4-1-like sequences.

Interestingly, drugs like procainamide and hydralazine that induce a lupus-like illness in genetically predisposed individuals are known to act as demethylating agents[16,29]. These drugs may promote autoimmunity by facilitating the transcription of endogenous viral antigens such as HERV antigens. Recent evidence indicates that mutation of the DNA methyltransferase gene can induce ICF immunodeficiency (immunodeficiency-centromeric instability-facial anomalies syndrome)[30]. Thus abnormalities of methylation-regulating genes seem to be related to the pathogenesis of immune dysfunction.

Our preliminary results indicate that 5-aza C mediated enhancement of transcription of clone 4-1-like sequences is related to 5-aza C induced less expression of mRNA of DNA methyltransferase-1 (DNMT-1; a kind of methylation-regulating enzyme) in controls, and the mRNA level of this DNMT-1 in SLE patients is lower than in healthy individuals (data not shown). It is possible that such abnormalities are associated with an increase of clone 4-1-like sequence

transcription in patients with SLE. Based on these findings, we are investigating the effect of drugs inducing a lupus-like illness on the transcription of clone 4-1, abnormalities of methylation-regulating genes in SLE, and the relationship between such abnormalities and SLE disease activity.

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