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Sequence Analysis of Genes Encoding Rodent Homologues of the Human Tumor-rejection Antigen SART-1

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Human *SART-1* (*hSART-1*) gene encodes a 125 kD protein with a leucine-zipper motif expressed in the nucleus of all proliferating cells, and a 43 kD protein expressed in the cytosol of most epithelial cancers. In this study, two rodent genes (*rSART-1* and *mSART-1*) homologous to *hSART-1* were cloned from cDNA libraries of murine brain and a rat tumor cell line, respectively. *mSART-1* and *rSART-1* were highly homologous to *hSART-1* with 86% and 84% identity at the nucleotide level, and 95% and 91% at the protein level, respectively. The leucine zipper domain and two basic amino acid portions that bind DNA, as well as peptide sequences recognized by human cytotoxic T lymphocytes (CTLs), were all conserved in these rodent genes. Nuclear protein homologous to the 125 kD hSART-1_{800} protein, but not to the 43 kD cytosol SART-1_{259} protein, was detectable with specific antibody in the nuclear fractions of rodent tumor cell lines, and normal rodent fetal liver and testis. These rodent genes should be a novel tool for studies on the biological roles of the *SART-1* gene, and also in the construction of animal models of specific immunotherapy using SART-1 gene products.

Key words: Tumor rejection antigen — Cytotoxic T lymphocyte — Rodent genes

A number of antigens recognized by HLA-class-I-restricted and tumor-specific cytotoxic T lymphocytes (CTLs) have recently been isolated,[1–6] raising the hope that they might be used to develop cancer vaccines for specific immunotherapy. Indeed, several peptides encoded by these genes are under clinical trial as cancer vaccines, and major tumor regression has been seen in melanoma patients.[7,8] We have recently identified a *SART-1* gene[9] encoding tumor antigens recognized by the CTLs[10] from cDNA of human esophageal cancer. The *SART-1* was suggested to be a bicistronic gene encoding two (125 kD and 43 kD) proteins. The 125 kD protein is expressed in the nucleus of proliferating cells, including normal and malignant cells, but not in non-proliferating cells, or in any normal tissues other than testis and fetal liver.[9] In contrast, the 43 kD protein is expressed in the cytosol of head and neck, esophageal and lung squamous cell carcinomas (SCC) and lung adenocarcinomas, but not in leukemia or melanomas, or in any normal tissues or cell lines other than fetal liver and testis.[9] The human bicistronic *LAP* gene has been shown to be involved in regulation of hepatocyte proliferation.[11] These results suggest that the *SART-1* gene is involved in cellular proliferation, although the mechanisms of this involvement are unknown. In this study, rodent genes homologous to *hSART-1* were cloned with the aim of better understanding the biological roles of the *SART-1* gene and also to provide animal models for specific immunotherapy with SART-1 gene products.

MATERIALS AND METHODS

Cloning of rodent *SART-1* genes A murine cDNA library was obtained from "SuperScript" Murine Brain cDNA Library in pCMV-SPORT 2 (GIBCO BRL, Gaithersburg, MD) and the rat cDNA library was prepared according to the manufacturer's instructions (GIBCO BRL). In brief, mRNA of the SCC-131 rat tumor cells was converted to cDNA, ligated to *Sal*I adapter, and inserted into the expression vector pSV-SPORT-1 (GIBCO BRL). The murine and rat *SART-1* homologue clones were obtained from the murine and rat cDNA plasmid libraries, respectively, by the colony hybridization method using ^{32}P-labeled *6A1-1D7*, a truncated human *SART-1* cDNA, as a probe.[9] Briefly, the cDNA library was plated out at approximately 100,000 colonies per screen onto nitrocellulose filters (NEN Research Products, Boston, MA) on agar plates, and cultured for 10 h. Replicate daughter filters were prepared and colonies were

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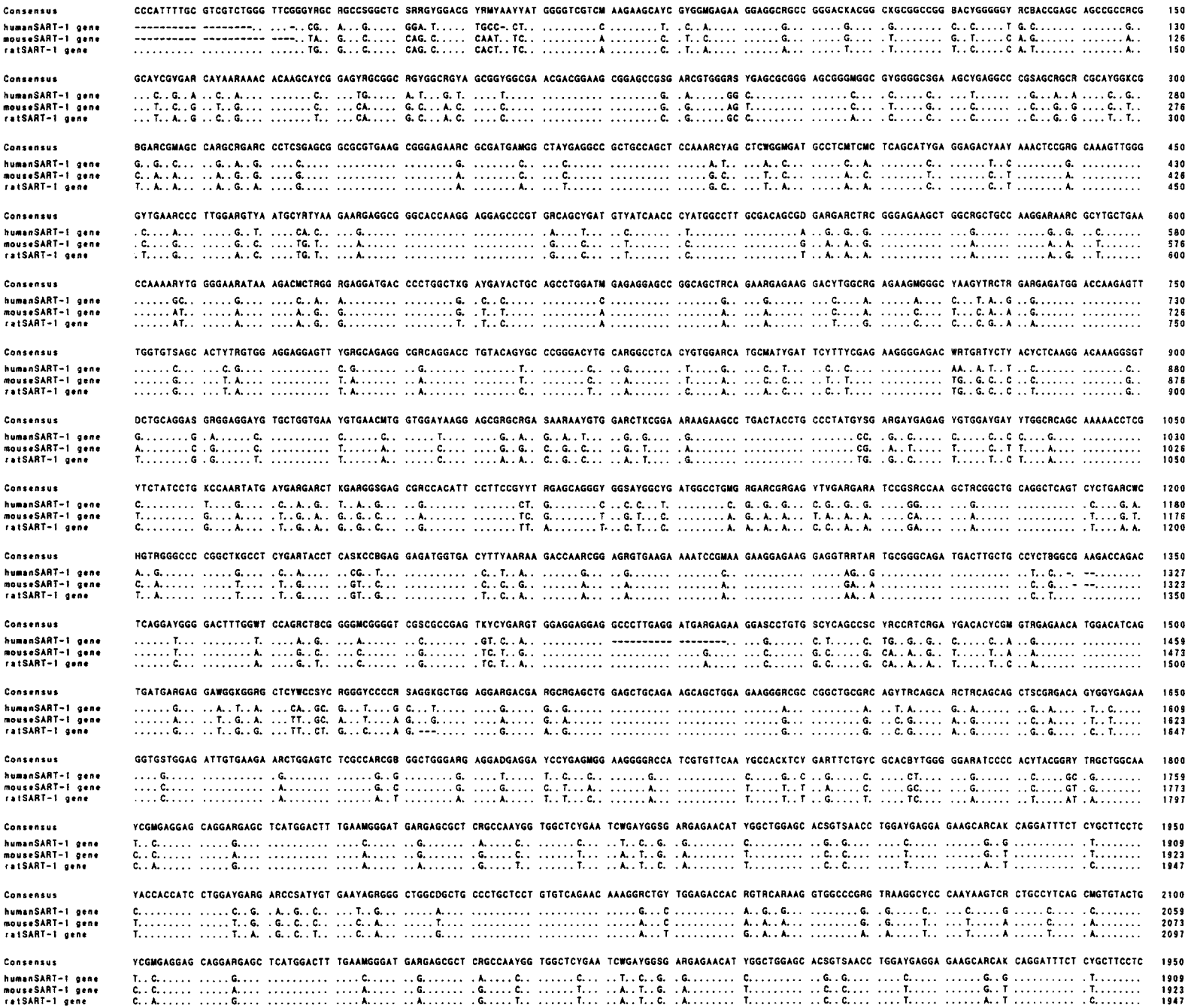
A



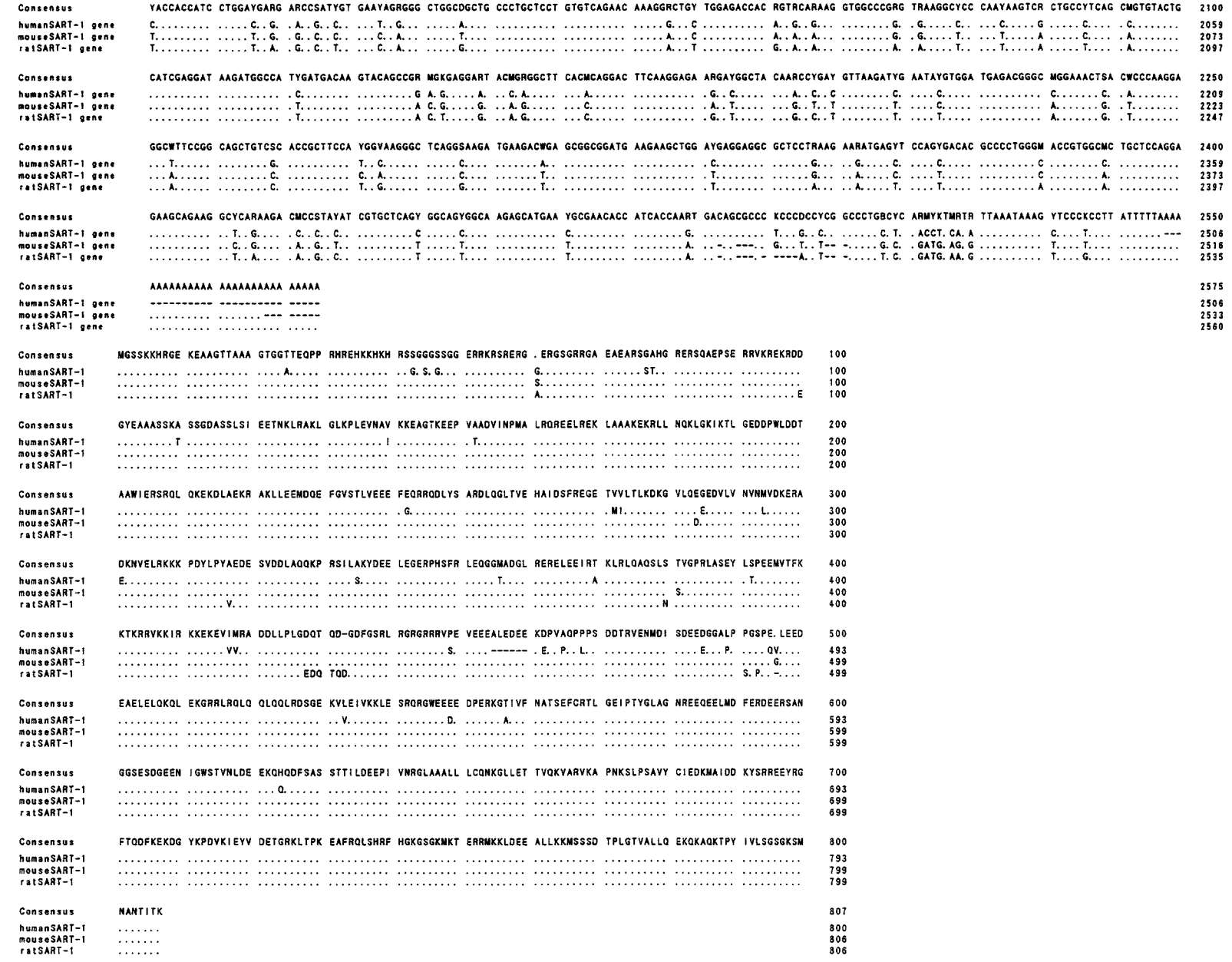


Fig. 1. Comparison of (A) nucleotide sequences between murine, rat, and human *SART-1* cDNA and (B) the predicted amino acid sequences. The murine and rat *SART-1* genes were cloned from murine and rat cDNA libraries, respectively. They consisted of 2,418 bp encoding a protein with 806 aa.

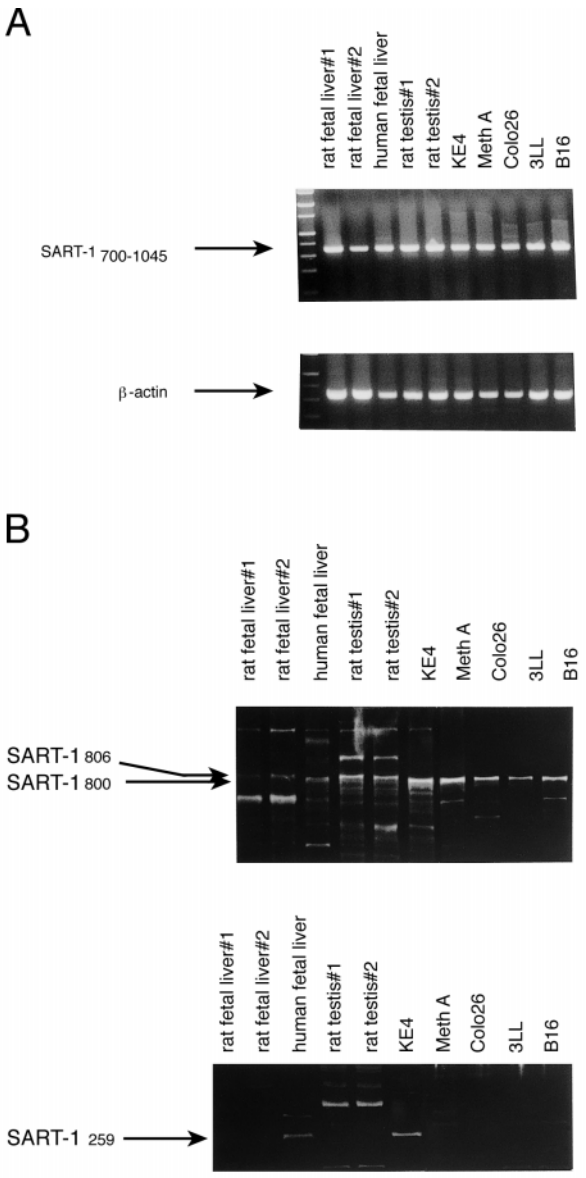


Fig. 2. Expression of *mSART-1* or *rSART-1* genes (A) at the mRNA level and (B) at the protein level. (A) Fetal rat (Wistar) livers (19 days), adult rat testis, liver, spleen, and kidney, 5 mouse cell lines, Colon 26 (colon cancer), 3LL (lung cancer), MN134 (hepatoma), Meth A (fibrosarcoma), B16 (melanoma), and 3 rat cell lines, SCC-131, SCC-158 (external auditory meatus squamous cell carcinoma), and KNRK (Kirsten sarcoma virus transformed normal kidney), were analyzed by the RT-PCR method. KE4 and human fetal liver were used as positive controls. Representative results are shown. (B) Polyclonal anti-SART-1_{800} recognizing the 125 kD hSART-1_{800} protein and polyclonal anti-SART-1_{259} recognizing the 43 kD hSART-1_{259} protein were used in western blot analysis as reported.[8] Both mSART-1_{806} and rSART-1_{806} were detected in the tumor cell lines, normal rat testis, and normal fetal rat liver, but not in adult rat liver, spleen, or kidney. KE4 and human fetal liver were used as positive controls. Representative results are shown.

lysed. After prehybridization for 3 h at 42°C in 50% (by vol.) formamide, hybridization was performed by adding to the prehybridization solution the denatured labeled *6A1-1D7* cDNA probe together with 10 μg/ml denatured salmon sperm DNA. The hybridization was carried out overnight at 42°C. The filter was washed twice at room temperature with 2× salt sodium citrate (SSC) and 0.2× SSC supplemented with 0.1% sodium dodecyl sulfate (SDS), followed by autoradiography at −80°C for approximately 5 h using BIOMAX (Kodak, Rochester, NY). Putative positive colonies were subjected to a second round of screening to facilitate the isolation of colonies. The largest clone of 2,513 or 2,532 base pairs was purified from the murine or rat cDNA library, respectively. DNA sequencing was performed using the dideoxynucleotide sequencing method with a DNA Sequencer Kit (Perkin Elmer, Applied Biosystems Division, Foster, CA) with an ABI "PRISM" 377 DNA Sequencer.

Expression of *SART-1* at the mRNA and protein levels

Samples used for the study were fetal rat (Wistar) livers from two prenatal rats in a 19-day pregnant rat, and adult rat testis, liver, spleen, and kidneys. Mouse cell lines, Colon 26 (colon cancer), 3LL (lung cancer), MN134 (hepatoma), Meth A (fibrosarcoma), and B16 (melanoma), and rat cell lines, SCC-131 and SCC-158 (external auditory meatus squamous cell carcinoma) were donated by the Japanese Cancer Research Resources Bank (JCRB, Tokyo), and KNRK (Kirsten sarcoma virus transformed normal kidney) was donated by American Type Culture Collection (ATCC, Rockville, MD). Tissues were sonicated for 60 to 90 s with an Astron ultrasonic processor (Heat Systems, Farmingdale, NY) before isolation of RNA. The *SART-1* mRNA expression in these tissues and cells was investigated by the reverse transcriptase-polymerase chain reaction (RT-PCR) method using specific primers (SART-1f 700: 5′-CCAAGTTACTGGAGGAGATGG-3′ and SART-1r1045: 5′-TTGGACAGGATAGAGCGAGG-3′). There was no risk of false positives due to small amounts of DNA contaminating the RNA preparation, since the primers corresponded to sequences located in different exons. Amplification was performed for 35 cycles of 1 min at 94°C, 2 min at 56°C and 2 min at 72°C. The detection of β-actin mRNA and the methods of the western blot analysis to detect the 125 kD SART-1_{800} protein and the 43 kD SART-1_{259} antigens were previously described.[9]

RESULTS

The nucleotide sequences of *mSART-1* and *rSART-1* are shown in Fig. 1A. Both are highly homologous to *hSART-1*, with 86% and 84% identity at the nucleotide level, respectively. The open-reading frames (ORF) of both *mSART-1* and *rRART-1* were 2,418 bp in length and

encoded a protein of 806 amino acids (aa). The predicted aa sequences are shown in Fig. 1B. The *mSART-1* sequence contains one small insertion at nt positions 1,388 to 1,405, resulting in an encoded protein of 806 aa, which is 6 aa (ALEDEE) longer than the human SART-1_{800} (hSART-1_{800}) protein. Similarly, *rSART-1* contains an insertion at nt positions 1,415 to 1,432, resulting in an encoded protein of 806 aa. hSART-1_{800} showed 95% and 91% homology with mSART-1_{806} and rSART-1_{806}, respectively. Both *mSART-1* and *rSART-1* encode a leucine zipper motif at around nt positions 1,114 to 1,198 (corresponding peptide, RELEEIRTKLRLQAQSLNTVGPRLAS) and at around nt positions 1,139 to 1,222 (corresponding peptide, RELEEIRTKLRLQAQSLSTVGPRLAS), respectively. Two basic aa-rich portions at aa positions 31 to 42 (RHREHKKHKHRS) and 400 to 414 (KKTKRRVKKIRKKEK) of the *hSART-1* were completely conserved in both *mSART-1* and *rSART-1*.

The rodent *SART-1* genes were expressed at the mRNA level in all samples tested (8 normal rat tissues, 2 fetal liver, 5 mouse cell lines, and 3 rat cell lines). Representative results are shown in Fig. 2A.

We then investigated whether the rabbit anti-hSART-1_{800} or anti-hSART-1_{259} polyclonal antibody (Ab) reacted to the rodent proteins corresponding to the hSART-1_{800} or hSART-1_{259} protein using western blot analysis. Anti-hSART-1_{800} Ab recognized the approximately 127 kD band of mSART-1_{806} or rSART-1_{806} in all the murine and rat tumor cell lines tested (SCC-131, SCC-158, B16, 3LL, MH134, Meth-A, KNRK, Colon 26) and in normal rat fetal liver and testis (Fig. 2B). The molecular weight was a little larger than that of hSART-1_{800}. In contrast, no band reactive to anti-hSART-1_{259} Ab was seen (Fig. 2B).

DISCUSSION

The nucleotide sequences of both *mSART-1* and *rSART-1*, as well as the predicted aa sequences, were all highly homologous to those of *hSART-1*. There were no significant differences among the human and rodent proteins in terms of hydrophobicity pattern analyses, such as Kyte-Doolittle hydropathy.[12] Both the *mSART-1* and *rSART-1* genes encode a leucine zipper motif that is highly homologous to that of *hSART-1* (at nt positions 1,119 to 1,202).[9] This leucine zipper motif is known to form a homo- or hetero-dimer that can bind DNA and modulate the transcription of many genes.[13] In fact, it has been shown that the *hSART-1* gene is expressed in the nucleus of cells at M-phase, and that it can bind DNA (Imai *et al*., unpublished data). Therefore, both *mSART-1* and *rSART-1* might also bind DNA and modulate the transcription of target genes. The basic aa domain that is capable of binding to DNA is often associated with this motif.[13] Two basic aa-rich portions of *hSART-1* were completely conserved in both *mSART-1* and *rSART-1*. This high homology between rodent and human indicates that the *SART-1* gene might play an important role at the M-phase with respect to the regulation of cellular proliferation, over a wide range of species.

The nucleotide sequences of *hSART-1* encoding antigenic peptides of human cancer cells were also well conserved in both *mSART-1* and *rSART-1*. These antigenic peptides encoded by *hSART-1* are SART-$1_{736–745}$ (KLDEEALLK) and SART-$1_{785–793}$ (VLSGSGKSM) recognized by the HLA-A26-restricted CTL,[9] and the SART-$1_{690–698}$ peptide (EYRGFTQDF) recognized by the HLA-A24-restricted CTL (Kikuchi *et al*., unpublished results). All three peptides are shared among the hSART-1_{259}, hSART-1_{800}, mSART-1_{806}, and rSART-1_{806}. The anchor residues of mouse class I, H-2K^d, were already reported as tyrosine (Y) or phenylalanine (F) at the 2nd position and isoleucine (I), leucine (L), or valine (V) at the 9th position of 9mer antigenic peptides.[14] Six different peptides with H-2K^d binding motifs were found in both mouse and rat SART-1 (aa positions 240–248, 265–273, 389–397, 398–406, 565–573, and 626–634). Our recent data have shown that one of them has the ability to induce MHC (major histocompatibility complex)-restricted and peptide-specific CTL in Balb/c mice (H-2K^d) (Yamaguchi *et al*., unpublished data). These results suggest that rodent *SART-1* genes might be a novel tool for developing animal models of specific immunotherapy with the SART-1 gene product.

The *SART-1* mRNA was ubiquitously expressed in all the rodent normal tissues and tumor cell lines tested. This is in agreement with the results from the northern blot analysis of human *SART-1* mRNA published previously.[9] We next investigated the expression of the rodent proteins corresponding to the hSART-1_{800} or hSART-1_{259} protein by western blot analysis with the rabbit anti-hSART-1_{800} or anti-hSART-1_{259} polyclonal Ab. Anti-hSART-1_{800} Ab recognized the 127 kD band of mSART-1_{806} or rSART-1_{806} in all the murine and rat tumor cell lines, and in normal rat fetal liver and testis. The molecular weights of these proteins were a little larger than that of hSART-1_{800}. In contrast, no band reactive to anti-hSART-1_{259} Ab was seen. There are two possible explanations for this failure. First, this Ab might not have recognized the rodent protein corresponding to hSART-1_{259}, since at least 4 out of 259 aa were different (at the aa positions 526, 542, 540, and 617 of the human peptide sequence). Secondly, the failure might be due to the fact that the start ATG at position 1,663–1,665 responsible for the hSART-1_{259} protein was not found in either *mSART-1* or *rSART-1*. Alternatively, rodent *SART-1* genes might not be bicistronic, since a Shine-Dalgarno (S-D) like sequence observed in *hSART-1* (AGG GGG at positions 1,681–1,686) was not found in either *mSART-1* (AGG GGA at positions 1,695–

1,700) or *rSART-1* (AGG GGA at positions 1,720–1,725). The S-D sequence is known to induce frameshifting in prokaryotic mRNAs, and is also suggested to be involved in frameshifting in some eukaryotic mRNAs.[15, 16]

In conclusion, these rodent genes should be a novel tool for studies on the biological roles of the *SART-1* gene, and also in the construction of animal models of specific immunotherapy using SART-1 gene products.

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