Sphingosine kinase-1 (SPHK1) is a key enzyme catalyzing the formation of an important bioactive lipid messenger, sphingosine 1-phosphate, and is implicated in the regulation of cell proliferation and anti-apoptotic processes. Biological features of another isozyme SPHK2, however, remain unclear. The present studies were undertaken to characterize SPHK2 by comparison with SPHK1. When SPHK2 was transiently expressed in various cell lines, it was localized in the nuclei as well as in the cytosol, whereas SPHK1 was distributed in the cytosol but not in the nucleus. We have mapped a functional nuclear localization signal (NLS) to the N-terminal region of SPHK2. We have observed that the expression of SPHK2 in various cell types causes inhibition of DNA synthesis, resulting in the cell cycle arrest at G1/S phase. We have also demonstrated that an NLS mutant of SPHK2, SPHK2R393E/R94E, failed to enter the nucleus and to inhibit DNA synthesis. Moreover, a fusion protein, NLS-SPHK1, where SPHK1 was fused to the NLS sequence of SPHK2 acquired the ability to enter nuclei and inhibited DNA synthesis. These results indicate that SPHK2 localizes in the nuclei and causes inhibition of DNA synthesis, and this may affect subsequent cellular events.

Sphingosine 1-phosphate (SPP) is a bioactive lipid that regulates diverse biological processes such as calcium mobilization, cell growth, differentiation, survival, motility, and cytoskeletal reorganization, acting both inside and outside the cells (1, 2). Recently, SPP was identified as the ligand for the differentiation gene-1 family, now collectively renamed SPP family of G protein-coupled receptors known as the endothelial differentiation gene-1 family (3–6), supporting a role for SPP as an extracellular messenger, sphingosine 1-phosphate, and is implicated in the phospholipid biosynthesis of an important bioactive lipid messenger, sphingosine 1-phosphate, and is implicated in the regulation of cell proliferation and anti-apoptotic processes. Biological features of another isozyme SPHK2, however, remain unclear. The present studies were undertaken to characterize SPHK2 by comparison with SPHK1. When SPHK2 was transiently expressed in various cell lines, it was localized in the nuclei as well as in the cytosol, whereas SPHK1 was distributed in the cytosol but not in the nucleus. We have mapped a functional nuclear localization signal (NLS) to the N-terminal region of SPHK2. We have observed that the expression of SPHK2 in various cell types causes inhibition of DNA synthesis, resulting in the cell cycle arrest at G1/S phase. We have also demonstrated that an NLS mutant of SPHK2, SPHK2R393E/R94E, failed to enter the nucleus and to inhibit DNA synthesis. Moreover, a fusion protein, NLS-SPHK1, where SPHK1 was fused to the NLS sequence of SPHK2 acquired the ability to enter nuclei and inhibited DNA synthesis. These results indicate that SPHK2 localizes in the nuclei and causes inhibition of DNA synthesis, and this may affect subsequent cellular events.

Sphingosine 1-phosphate (SPP) is a bioactive lipid that regulates diverse biological processes such as calcium mobilization, cell growth, differentiation, survival, motility, and cytoskeletal reorganization, acting both inside and outside the cells (1, 2). Recently, SPP was identified as the ligand for the differentiation gene-1 family, now collectively renamed SPP family of G protein-coupled receptors known as the endothelial differentiation gene-1 family (3–6), supporting a role for SPP as an extracellular messenger, sphingosine 1-phosphate, and is implicated in the phospholipid biosynthesis.
 GCC ACC ATG TAC CCA TAC GAT GAA ATC ACC CCT GAA TTG CTG 3' and 5'-AGA GGT ACC CAA GGC TGG TTC TGT GGT AG 3' sense and antisense primers, respectively.

NLS-mSPHK1 expression vector was prepared by PCR amplification using pTB-701-HA-mSPHK1-GFP as a template with sense primer 5'-GCA AGA TCT CGT GGC CGT CGA GGG GGC CGG CGC AGA GCT ACG CGG ATG GAA CCA GAA TGC 3', which contains the putative NLS region (residues 87–98) of SPHK2 and antisense primer 5'-GCA AGA TCT CGT CGA GGG GGC CG 3'. Site-directed mutagenesis for mSPHK2R93E/R94E was performed using mutagenic synthetic oligonucleotides (5'-GC CGT CGA GGG GGC GAG GAG AGA GCT ACG CGG ACC 3' and its reverse complement) and pTB-701-HA-mSPHK2-GFP as a template with a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All of the constructs were verified by DNA sequencing.

The HA-mSPHK2-GFP cDNA was also subcloned into pUHG10-3 plasmid for the secondary stable transfection. The HeLa Tet-On cells (Clontech) were co-transfected with the plasmids pUHG10-3-HA-mSPHK2-GFP and pSV2her (Kaken Pharmaceutical Co., Tokyo). Drug-resistant clones were isolated in the presence of 2.5 μg/ml blastocidin. From the blastocidin-resistant clones, the cells harboring the pUHG10-3-HA-mSPHK2-GFP were selected on the basis of the highest inducibility of HA-mSPHK2-GFP expression in the presence of 100 ng/ml doxycycline (Dox).

Immunocytochemistry—Cells were grown on four-chambered slides (Nalge/Nunc) and transfected using Fugene-6 reagent according to the manufacturer's instructions (Roche Applied Science). Subcellular localization studies using confocal microscopy were performed as described previously (11). A rabbit polyclonal anti-hSPHK2 antibody was raised against the synthetic peptide SQALQHIQRLRPKPEARPR (amino acid residues 35–52) conjugated to keyhole limpet hemocyanin. The antibody was further purified by the immunogen peptide-immobilized Sepharose 4B.

Isolation of Nuclei—The separation of nuclei from cells was carried out essentially as described (13), with some modifications (14). For the preparation of cytosolic fraction, the supernatant fractions above the
sucrose cushion obtained after 15,000 × g step were further centrifuged at 100,000 × g for 30 min. The resultant nuclear and cytosolic fractions were assayed for enzymatic activity. For immunoprecipitation studies, nuclear fractions were obtained as described (15).

Measurement of in Vitro SPHK Activity—SPHK activity was determined in the presence of sphingosine, prepared as a complex with 4 mg/ml bovine serum albumin and (γ−32P)ATP in kinase buffer containing 50 mM KCl as described previously (8) using either cell extracts or purified nuclear fractions as an enzyme source. In Fig. 2, the nuclear fractions were purified from HeLa cells grown in 10-cm dishes as described in Ref. 15 and by lysed by sonication in cold lysis buffer (20 mM Tris-HCl, pH 7.4, 130 mM NaCl, 1% (w/v) Triton X-100, and protease inhibitors (Roche Applied Science)). The nuclear lysates were clarified by centrifugation for 15 min at 10,000 × g and incubated for 1 h with anti-SPHK2 antibody (9 μg). The immunoprecipitates were collected by protein A/G Plus-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and then washed three times with the lysis buffer and used as an enzyme source. [32P]SPP was separated by thin layer chromatography and quantitated using a Fujix Bio-Imaging Analyzer BAS 2000 (Fuji Photo Film).

Measurement of DNA Synthesis—Bromodeoxyuridine (Brdurd) incorporation was measured using Brdurd labeling and detection kit I (Roche Applied Science). Briefly, NIH 3T3 cells grown on chambered slides were transiently transfected with either the GFP control plasmid or various plasmid constructs as indicated in Fig. 5 legend. Two days after transfection, cells were incubated for 5 h with 10 μM Brdurd and then fixed in 70% ethanol containing 50 μM glycine (pH 2.0) for 20 min at 4 °C. Washing with PBS, cells were incubated for 1 h at 37 °C with mouse monoclonal anti-Brdur and rat monoclonal anti-HA antibodies. After washing with the washing buffer, cells were incubated for 1 h with Alexa 594-conjugated goat anti-mouse IgG and Alexa 488-conjugated goat anti-rat IgG in PBS containing 0.1% bovine serum albumin. Five random fields from each sample were analyzed with a confocal microscope. The percentage of transfected cells (green HA fluorescence) incorporating Brdurd into DNA was calculated as an average of four sample slides with a minimum of 100 cells scored per field.

For [3H]thymidine incorporation studies, HeLa cells stably expressing SPHK2 were seeded in six-well plates at a density of 105 cells/well in Dulbecco's modified Eagle's medium supplemented with 10% FCS with various concentrations of Dox as specified in the figure legends. After 24 h, the serum in the medium was reduced from 10 to 0.5%, while the medium was supplemented with 2.5 mM thymidine for 19 h. Cells were washed twice with PBS and maintained further in Dulbecco's modified Eagle's medium with 10% FCS and 2.5 mM thymidine for 19 h. Cells were washed twice with PBS and maintained further in Dulbecco's modified Eagle's medium with 10% FCS for 10 h. Cells were treated again with 2.5 mM thymidine for 10 h at room temperature. The cells were washed with PBS, resuspended in ice-cold 70% ethanol in PBS, and incubated for 10 min. The cells were washed once with PBS and resuspended in PBS containing 50 μg/ml propidium iodide (PI) and 125 units/ml ribonuclease A and incubated for 1 h at room temperature in the dark. Flow cytometric analysis for GFP and PI fluorescence was performed using a four-color FACS Calibur (BD Biosciences). Electronic compensation was used among the fluorescence channels to remove residual spectral overlap. GFP and PI fluorescence data were collected on a logarithmic and a linear scale, respectively. For each sample, 40,000 events were collected. Analyses of the multivariate data and DNA histograms were performed with CELL QUEST and ModFITLTD software, respectively (Verity Software House, Topsham, ME) that are part of the FACS Calibur operating system.

Other Procedures—Lactate dehydrogenase activity was measured according to the supplier's protocol (Kyokuto Chemicals, Tokyo). Protein was determined by the method of Bradford (18).

### Table I

| Cell types and density | Cell populations expressing SPHK2 |
|------------------------|----------------------------------|
|                        | Control | Low (104/mm2) | High (3 × 106/mm2) |
| HeLa cells (low       | 82.9 ± 2.0 | 12.2 ± 0.6 | 4.9 ± 2.5 |
| confuence)            |         |             |               |
| HeLa cells (high       | 88.7 ± 1.9 | 10.0 ± 1.3 | 1.3 ± 0.6 |
| confuence)            |         |             |               |
| COS7 cells (low        | 61.4 ± 2.2 | 24.5 ± 2.8 | 14.1 ± 3.0 |
| confuence)            |         |             |               |
| COS7 cells (high       | 10.3 ± 1.8 | 17.1 ± 1.3 | 72.7 ± 1.3 |
| confuence)            |         |             |               |
| HER293 cells (low      | 7.5 ± 1.1 | 9.6 ± 1.2 | 82.6 ± 2.0 |
| confuence)            |         |             |               |
| HER293 cells (high      | 4.6 ± 1.5 | 9.2 ± 2.5 | 86.2 ± 3.8 |
| confuence)            |         |             |               |

**RESULTS**

**SPHK2 Is Predominantly Localized in the Nucleus**—During immunocytochemical analyses of SPHK2, we observed that SPHK2 was predominantly localized in the nuclei of various cell types. When mouse SPHK2 was fused with GFP and transiently expressed in COS7 cells, mSPHK2-GFP was mainly localized in the nuclei and to a lesser extent in the cytoplasm (Fig. 1A). Nuclear localization of SPHK2 was also observed when it was transiently expressed in HeLa cells (Fig. 1B) and NIH 3T3 cells (Fig. 1C). To exclude the possibility that GFP fusion itself had caused SPHK2 to mislocalize into the nuclei and to show that the nuclear localization of SPHK2 is a general characteristic independent of species differences, human SPHK2 (without any added tag sequence) was transiently expressed in HeLa cells, and its intracellular distribution was examined using a specific antibody raised against a peptide sequence common to both human and mouse SPHK2. Similar to mSPHK2, hSPHK2 was mainly localized in the nuclei with some cytoplasmic staining in these cells (Fig. 1D). The nuclear localization of hSPHK2 was confirmed by the nucleus-specific 4,6-diamidino-2-phenylindole (DAPI) staining of HeLa cells (Fig. 1E) and the colocalization of DAPI with hSPHK2 staining (Fig. 1F). Next, the other SPHK isoforms, SPHK1, was fused with GFP, and its intracellular distribution was compared with SPHK2. In COS7 cells, mSPHK1-GFP was localized mainly in the cytoplasm but not in the nucleus (Fig. 1H), an observation that is consistent with a previous report (9).

It is important to test whether the nucleo-cytoplasmic distribution of SPHK2 may change depending on cell type and cell confluence. When hSPHK2 was transiently expressed in HeLa cells, it was predominantly localized in the nuclei under all tested conditions (Table I). In the case of COS7 cells, the intracellular distribution of hSPHK2 was dramatically influenced by cell density; nuclear localization was prominent (61.4%) particularly in high cell density cultures, whereas it was only 10.3% in low cell density cultures. In contrast, hSPHK2 was predominantly localized in the cytosol irrespective of cell density in HEK293 (Table I).

Next, we tried to determine whether endogenous SPHK2 was also localized in the nucleus similar to exogenously-expressed SPHK2. In HEK293 cells, mSPHK2-GFP was mainly localized in the nuclei with some cytoplasmic staining in these cells (Fig. 1D). The nuclear localization of mSPHK2 was confirmed by the nucleus-specific 4,6-diamidino-2-phenylindole (DAPI) staining of HEK293 cells (Fig. 1E) and the colocalization of DAPI with mSPHK2 staining (Fig. 1F). Next, the other SPHK isoform, SPHK1, was fused with GFP, and its intracellular distribution was compared with mSPHK2. In COS7 cells, mSPHK1-GFP was localized mainly in the cytoplasm but not in the nucleus (Fig. 1H), an observation that is consistent with a previous report (9).
pressed recombinant SPHK2. To address this issue, endogenous SPHK2 from purified nuclear extracts of HeLa cells was immunoprecipitated with anti-hSPHK2 antibody and analyzed for its activity. The immunoprecipitates showed clear SPP production, whereas the immunoprecipitates prepared in the presence of the immunogen peptide contained no detectable radioactivity corresponding to SPP, suggesting that the purified nuclear fractions contained SPHK activity that bound specifically to the antibody (Fig. 2A). The aliquots of the immunoprecipitates prepared in the absence of the immunogen peptide were further analyzed by immunoblot using anti-hSPHK2 antibody (Fig. 2B). The immunoprecipitates from nuclear fractions revealed a clear band around 70 kDa, which corresponded to the position of affinity-purified recombinant HA-hSPHK2. The immunoreactive band around 70 kDa sometimes appeared as a doublet, suggesting the existence of post-translational modification of the protein. The anti-hSPHK2 antibody did not cross-react with HA-SPHK1. When the immunoprecipitates were immunoblotted with anti-SPHK1 antibody, there was no immunoreactive band either around authentic HA-SPHK1 or HA-SPHK2. These results strongly suggest that the anti-hSPHK2 antibody specifically recognizes endogenous SPHK2 but not SPHK1 and that the purified nuclear fractions contain endogenous SPHK2. This conclusion was further confirmed by immunocytochemical analyses. After fixation and staining of HeLa cells with anti-hSPHK2 antibody, endogenous SPHK2 was found to be distributed mainly in the nucleus and to a lesser extent in the cytosol (Fig. 2C), which is consistent with

**Fig. 2. Detection of endogenous SPHK2.** A, detection of endogenous SPHK2 activity. Endogenous SPHK2 from the purified nuclear lysates from HeLa cells was immunoprecipitated (IP) using anti-hSPHK2 antibody in the absence or presence of 15 μg/ml immunogen peptide. The immunoprecipitates were washed and assayed for SPHK activity. The endogenous SPHK2 produced 1.4 pmol of SPP/min/tube. Contamination of the purified nuclear fractions from cytosolic, endosomal, or endoplasmic reticulum fractions was estimated to be less than 3, 3, and 9%, respectively, as judged by the content of several specific protein markers: lactate dehydrogenase for cytosol, early endosomal antigen 1 for endosomes, and BiP/GRP78 for endoplasmic reticulum (see Supplemental Material). B, the aliquots of immunoprecipitates without immunogen peptide, affinity-purified HA-SPHK1, and HA-SPHK2 were subjected to 12.5% SDS-PAGE followed by immunoblot analyses using anti-hSPHK2 or anti-SPHK1 antibody (Abcam, Cambridge, UK). The molecular masses of standard proteins and the position of heavy and light chains of immunoglobulin are indicated. The arrows mark the positions of SPHK1 and SPHK2. C, HeLa cells were fixed, permeabilized, and stained for confocal microscopy analyses using anti-hSPHK2 antibody (red). Nuclei were also stained with 2 μg/ml DAPI (blue). Bar, 10 μm.

**Fig. 3.** The NLS of SPHK2 resides in the N-terminal portion of the protein. A, a schematic representation of conserved regions of mSPHK1, mSPHK2, and deletion mutants of mSPHK2. B, comparison of NLS sequences in mSPHK2 and other proteins. The positions and sequences of the putative NLS regions from mSPHK2, Rev protein, Rex protein, Tat protein, and mPRCA are shown in relation to a diagram of the full-length mSPHK2 protein. The basic amino acid residues known to be important for NLS function (arginine and lysine) are shown in *boldface* type. Amino acid sequences are aligned based on the position of these basic amino acid residues. The numbers indicate the location of the amino acids within the respective proteins. For comparison, a consensus sequence of the NLS is shown. The *asterisks* indicate the arginine residues in SPHK2 that were mutated to glutamate residues in the current study.
the findings observed using a recombinant overexpressed system (Fig. 1 and Table I).

NLS Is Localized in the N-terminal Portion of SPHK2—mSPHK2 encodes a protein, which is larger than mSPHK1 and contains an additional 236 amino acids (8). Compared with SPHK1, SPHK2 contains two largely unrelated sequences, one at the amino terminus and the other in the middle of the protein (Fig. 3A). It may not be unreasonable to assume that the differences in the intracellular distribution between mSPHK1 and mSPHK2 are attributable to these additional unrelated sequences, which exist only in mSPHK2. To test this possibility, we constructed two deletion mutants: mSPHK2ΔM, which lacks the middle portion of the unrelated sequence, and mSPHK2ΔN, which lacks the amino-terminal portion of the protein (Fig. 3A). When mSPHK2ΔM-GFP was transiently expressed in COS7 cells, the protein was localized mainly in the nuclei showing that the middle portion of SPHK2 had no influence on the nuclear localization of this protein (Fig. 1J). On the other hand, mSPHK2ΔN-GFP could not enter the nuclei when it was expressed in COS7 cells (Fig. 1K). These results strongly suggest that an NLS sequence resides within the amino terminus of mSPHK2.

Identification of the NLS Sequence of SPHK2—Although the sequence of mSPHK2 has been analyzed for its domain structure, so far no one has reported the presence of an NLS identical to the previously known NLS sequences. However, our analyses showed that SPHK2 possesses a putative NLS (RGRGGRRR) with an arginine cluster, which is highly similar to a type of monopartite NLS enriched in arginine residues identified in the Tat and Rev proteins of human immunodeficiency virus type 1 (19), the Rex protein of human T-cell leukemia virus type 1 (20), and the atypical protein kinase C λ (21) (Fig. 3B). To demonstrate that this putative NLS functions as a true NLS in the cells, another mutant mSPHK2R93E/R94E-GFP, where both Arg-93 and Arg-94 of mSPHK2-GFP were mutated to glutamic acid, was constructed and analyzed. A similar mutation in the NLS sequence of protein kinase Ca has been reported to abolish the nuclear accumulation of protein kinase Ca (21). When mSPHK2R93E/R94E-GFP was transiently expressed in COS7 cells, the mutant protein could not enter the nuclei (Fig. 1L), indicating that these arginine residues were critical for the functioning of this NLS.

To show that this tentative NLS sequence of SPHK2 functions as a true NLS, this sequence was fused with a cytoplasmic protein, SPHK1, and its nuclear targeting efficiency was analyzed. When NLS-mSPHK1-GFP was transiently expressed in COS7 cells, the protein accumulated mainly in the nuclei (Fig. 1M). These observations indicate that the NLS sequence of mSPHK2 was necessary and sufficient to direct a complete nuclear import of SPHK2.

SPHK2 Causes Inhibition of DNA Synthesis—Nuclear localization of SPHK2 as observed in Figs. 1 and 2 raised the possibility that this enzyme may be involved in some nuclear function. We next analyzed DNA synthesis as measured by [3H]thymidine incorporation into cells stably expressing SPHK2. For these experiments, HA-mSPHK2-GFP was stably expressed in HeLa Tet-On cells in a Dox-inducible manner. The cells expressing SPHK2 (induced by 0.1 μg/ml Dox) showed a 50% inhibition in [3H]thymidine incorporation compared with the cells expressing no exogenous SPHK2 under Dox-deficient conditions (Fig. 4A). Dox-inducible expression of SPHK2 was confirmed by immunoblot analyses using anti-HA antibody as shown in Fig. 4B. In the cells carrying empty vector, Dox at the concentrations used here had no significant effect on thymidine incorporation (data not shown).

Nuclear Localization Is Necessary for SPHK2 to Inhibit DNA Synthesis—The mechanism of the inhibition of DNA synthesis by SPHK2 was further assessed by measuring BrdUrd incorporation into nascent DNA in NIH 3T3 cells transiently expressing various constructs of SPHK and its mutants. When HA-mSPHK2-GFP was expressed in NIH 3T3 cells, BrdUrd incorporation was strongly inhibited as compared with the cells transfected with a control vector (Fig. 5). In contrast, cells expressing HA-mSPHK1 showed a 40% increase in BrdUrd incorporation, in agreement with a previous report (9). It is important to determine whether the nuclear localization of SPHK2 is required for the inhibition of DNA synthesis. The next experiments were designed to show the importance of nuclear localization of SPHK2 for inhibition of DNA synthesis by manipulating the ability of nuclear localization of SPHK2. Upon transient transfection in NIH 3T3 cells, the NLS mutant, HA-mSPHK2R93E/R94E, which failed to enter the nucleus (Fig. 1L), lost its ability to inhibit DNA synthesis (Fig. 5). More importantly, when mSPHK1 acquired nuclear localization ability upon fusion with the NLS sequence from mSPHK2, it showed inhibitory effects on DNA synthesis, suggesting that nuclear localization, and not the other features of SPHK2, is important for the inhibition of DNA synthesis. When hSPHK2 without any added tag sequences was transiently expressed in HeLa cells, essentially the same inhibitory effect on BrdUrd
uptake was observed (data not shown).

Next, we have checked the protein expression and enzymatic activity of various SPHK constructs. As shown in Fig. 6A, vector-transfected COS7 cells have low levels of SPHK activity. In cells transfected with HA-mSPHK1, NLS-HA-mSPHK1, or hSPHK2, in vitro SPHK activity was increased by 63-, 53-, and 52-fold, respectively. We have further demonstrated the in vitro accumulation of SPP in the nucleus using purified intact nuclear fractions. HeLa cells transiently expressing various SPHK proteins were disrupted, and the nuclei were isolated through high sucrose solution (13). In cells expressing hSPHK2, SPP accumulated mostly in the nuclear fractions (75%) (Fig. 6B), which supports the results obtained from the morphological determinations (Table I). On the other hand, SPP accumulated mainly in the cytosol of cells expressing HA-mSPHK1, consistent with a previous report (9). NLS-HA-mSPHK1 showed a clear shift in nuclear accumulation of SPP, confirming the results from the confocal microscopic analyses (Fig. 1M). In all nuclear preparations, lactate dehydrogenase, a typical cytosolic marker protein, was almost undetectable, whereas the typical nuclear membrane marker nucleoporin was detected exclusively in the nuclear fractions, validating the reliability of the cell fractionation technique.

**SPHK2 Expression Causes Cell Cycle Arrest at G1/S Phase**—

Next, the effect of SPHK2 on cell cycle was studied. NIH 3T3 cells transiently transfected with mSPHK2-GFP were synchronized to the beginning of the S phase by a double thymidine block, and the DNA content of cells expressing the SPHK2 protein was analyzed by flow cytometry. About 100% of the nonexpressing and SPHK2-expressing cells had not entered G2/M phase at 0 h as a result of double thymidine block-induced synchronization of the cell cycle (Fig. 7B). Upon release from the thymidine block, the percentage of cells in various phases of the cell cycle remains very different in the two populations. In the SPHK2-nonexpressing cells, a majority of cells entered the S phase after 3 h, and the bulk of the cells left the S phase and entered the G2/M phase by 9 h (Fig. 7B). In contrast, the SPHK2-expressing cells lagged in their progression through the cell cycle and persisted in the G1 phase much longer, showing a typical G1/S arrest, such that at the 9-h time point when nearly 40% of the nonexpressing cells were in the G2/M, only a very modest 7% of the SPHK2-expressing cells were in the G2/M phase of the cell cycle. NIH 3T3 cells expressing GFP alone showed a pattern essentially similar to the nonexpressing cells as in Fig. 7B (data not shown). This G1/S arrest eventually did not lead to apoptosis as judged by the occurrence of cells in the sub-G1 region (hypodiploidy) in cell cycle analysis. The results from flow cytometric analyses strongly suggest that SPHK2 causes inhibition of DNA synthesis through cell cycle arrest at G1/S phase.

**DISCUSSION**

This is the first report to show that SPHK2 is predominantly localized in the nuclei (Figs. 1 and 2), depending on the cell type.
and cell confluence (Table I). The present results as summarized in Table I are also consistent with a previous report that SPHK2 transiently transfected in HEK293 cells mainly localizes in the cytosol (8). However, in HeLa cells, hSPHK2 is almost exclusively in the nucleus, and in COS7 cells it is dominantly nuclear in high cell density cultures. Whether SPHK2 is involved in contact inhibition of proliferation in COS7 cells is an attractive possibility that needs to be explored carefully. Molecular mechanisms underlying the regulation of nucleo-cytoplasmic shuttling of SPHK2 are at present unknown and need to be studied using various model cell systems. Recently, Kleuser et al. (14) have reported that platelet-derived growth factor stimulates nuclear SPHK activity in Swiss3T3 cells. They have suggested that SPHK1 translocates from the cytosol to the nuclear envelope upon stimulation of cells by platelet-derived growth factor. However, the present data dem-
onstrate that it is SPHK2, which is localized mainly in the nucleoplasm and not in the nuclear envelope (Fig. 1).

We have also demonstrated that nuclear localization of SPHK2 causes inhibition of the DNA synthesis (Figs. 4 and 5). That this observation is not an artifactual effect caused by overexpressing exogenously introduced genes but represents an intrinsic feature of SPHK2 is supported by several arguments. First, the inhibition of DNA synthesis is specific to SPHK2 expression but not to a control, empty vector or SPHK1 overexpression (Fig. 5). Second, the inhibitory effect of SPHK2 overexpression on DNA synthesis was cancelled by introducing the NLS mutant (Fig. 5), although the levels of expression of the expression on DNA synthesis was cancelled by introducing the NLS mutant (Fig. 5), although the levels of expression of the

ments. First, the inhibition of DNA synthesis is specific to SPHK2 expression but not to a control, empty vector or SPHK1 overexpression (Fig. 5). Second, the inhibitory effect of SPHK2 overexpressing exogenously introduced genes but represents an proper understanding of the molecular mechanism of proliferation, differentiation, and apoptosis.

Acknowledgments—We thank Dr. Y. Nishizuka for helpful and critical discussion. We also thank M. Honma for skillful secretarial assistance.

REFERENCES

1. Le Stunff, H., Peterson, C., Liu, H., Milstien, S., and Spiegel, S. (2002) Biochim. Biophys. Acta 1582, 1–17
2. Pyne, S., and Pyne, N. J. (2002) Biochim. Biophys. Acta 1582, 121–131
3. Pyne, S., and Pyne, N. J. (2000) Biochem. J. 349, 385–402
4. Goetzl, E. J., and An, S. (1998) FASEB J. 12, 1589–1598
5. Spiegel, S., and Milstien, S. (2000) Biochim. Biophys. Acta 1484, 107–116
6. Hla, T., Lee, M. J., Ansell, N., Paik, J. H., and Kluk, M. J. (2001) Science 294, 1875–1878
7. Kohama, T., Olivera, A., Edsall, L., Nagiec, M. M., Dickson, R., and Spiegel, S. (1998) J. Biol. Chem. 273, 23722–23728
8. Liu, H., Sugiura, M., Nava, V. E., Edsall, L. C., Kono, K., Poulton, S., Milstien, S., Kohama, T., and Spiegel, S. (2000) J. Biol. Chem. 275, 19513–19520
9. Olivera, A., Kohama, T., Edsall, L., Nava, V., Cavillier, O., Poulton, S., and Spiegel, S. (1999) J. Cell Biol. 147, 545–558
10. Xia, P., Wang, L., Moretti, P. A., Albanese, N., Chai, F., Pitson, S. M., D’Andrea, R. J., Gamble, J. R., and Vadas, M. A. (2002) J. Biol. Chem. 277, 7996–8003
11. Hayashi, S., Okada, T., Igarashi, N., Fujita, T., Jahanseer, S., and Nakamura, S. (2002) J. Biol. Chem. 277, 33319–33324
12. Lacanà, E., Maczyka, M., Milstien, S., and Spiegel, S. (2002) J. Biol. Chem. 277, 32947–32953
13. Bunce, C. M., Thick, J. A., Lord, J. M., Mils, D., and Brown, G. (1988) Anal. Biochem. 176, 67–73
14. Kleuser, B., Maczyka, M., Milstien, S., and Spiegel, S. (2001) FEBS Lett. 503, 85–90
15. Chauveau, J., Moulé, Y., and Rouiller, C. (1996) Exp. Cell Res. 11, 317–321
16. Olivera, A., and Spiegel, S. (1995) Nature 365, 557–560
17. Krek, W., and DeCaprio, J. A. (1995) Methods Enzymol. 254, 114–124
18. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
19. Truant, B. R. (1999) Mol. Cell Biol. 19, 1210–1217
20. Palmeri, D., and Malim, M. H. (1999) Mol. Cell Biol. 19, 1218–1225
21. Perander, M., Björkoy, G., and Johansen, T. (2001) J. Biol. Chem. 276, 13015–13024
