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A Novel Homologue of the *TIAP/m-survivin* Gene

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The inhibitor of apoptosis (IAP) proteins comprise a highly conserved gene family that prevents cell death in response to a variety of stimuli. TIAP/m-survivin, a murine homologue of human Survivin, is a member of the IAP family. TIAP/m-survivin has one baculovirus IAP repeat (BIR) and lacks a C-terminal RING finger motif. Here we identified the genomic DNA region (TIAP-2) that is homologous to the *TIAP/m-survivin* gene by a low stringency genomic DNA hybridization. The region is on the chromomsome 9 which is distinct from that (chromosome 11) of the *TIAP/m-survivin* gene, and contains DNA sequence similar to a part of the BIR and the 3′ side of the *TIAP/m-survivin* gene and the sequence homology between them is 92%. Expression of *TIAP-2* mRNA was detected in various murine tissues by RT-PCR. Although expression of *TIAP/m-survivin* mRNA is upregulated in synchronized cells at S to G2/M phase of the cell cycle, expression of *TIAP-2* mRNA was constant in the cell cycle, suggesting the different role of TIAP-2 from that of TIAP/m-survivin. © 2001 Academic Press

Apoptosis, or programmed cell death, plays a major role in development, tissue homeostasis and defense against infectious agents (1). Dysregulated apoptosis contributes to many pathologies, including tumor promotion, autoimmune and immunodeficiency diseases, and neurodegenerative disorders (2). Apoptosis is mediated by members of the caspase family of proteases, and eventually causes the degradation of chromosomal DNA (3). The caspases are present as proenzymes in viable cells and are proteolytically processed to generate active forms in apoptotic cells. Those active caspases cleave an inhibitor of caspase-activated deoxyribonuclease (ICAD) and allow CAD to enter the nucleus and degrade chromosomal DNA (4). The counteraction of caspases by apoptosis inhibitory proteins, such as the Bcl-2 family and inhibitor of apoptosis (IAP) protein family, are crucial regulators in the molecular mechanism of apoptosis.

The IAP proteins comprise a highly conserved gene family that prevents cell death in response to a variety of stimuli. IAP was first identified in baculovirus genes that can complement the loss of the caspase inhibitor, p35, in mutant viruses (5–7). Cellular homologues of IAPs have also been noted in yeast (8), Drosophila (9), and mammals (10–17). In mammals, NAIP (10), c-IAP1 (HIAP2/MIHB), c-IAP2 (HIAP1/MIHC/hITA) (11–14), XIAP (hILP) (12, 13), BRUCE (15), pIAP (16), and Survivin (17) have been isolated. All of the *IAP* genes isolated from different species have the common structure termed the baculovirus IAP repeat (BIR). Recent studies showed that four mammalian IAPs, XIAP, c-IAP1, c-IAP2, and Survivin, bind to specific cell death proteases, caspase 3 and caspase 7, and inhibit their proteolytic activity *in vitro* (18–21).

We have described a newly defined murine IAP, designated TIAP that proved to be a murine homologue of human Survivin (22). TIAP/mouse survivin (m-survivin) has one baculovirus IAP repeat, and interacts with the processed form of caspase 3 and inhibits caspase-induced cell death. Histological examinations revealed that TIAP is expressed in growing tissues such as thymus, testis, and intestine of adult mice, and many tissues of embryos. Furthermore, expression of TIAP is upregulated in synchronized cells at S to G2/M phase of the cell cycle (22, 23). Survivin is also expressed in G2/M phase of the cell cycle in a cycle-regulated manner, and associates with microtubules of the mitotic spindle in a specific and saturable reaction that is regulated by microtubule dynamics (24). TIAP/m-survivin is, therefore, a newly identified member of the growing *IAP* gene family coding for caspase inhibitors and may be involved in molecular mechanisms of apoptosis during cell proliferation. Since the *IAP* genes compose a gene family, we tried to identify the TIAP/m-survivin homologous gene(s) by a low stringency

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genomic DNA hybridization with various parts of the *TIAP/m-survivin* gene as a probe. Here we show a homologous DNA region (TIAP-2) that was ubiquitously transcribed in various murine tissues. We discuss the presence of *TIAP-2* gene as a member of TIAP/m-survivin family.

MATERIALS AND METHODS

Animals. C57BL/6 mice were purchased from Japan SLC Co., Ltd. (Hamamatsu, Japan).

Southern blot analysis. Southern blot analysis was done as described elsewhere (25). Briefly, genomic DNAs digested with various restriction enzymes were separated on a 1% agarose gel and transferred onto a nylon membrane (Amersham, Buckingkamshire, England). The filter was hybridized overnight with the digoxigenin (DIG)-labeled probe at 42°C. Following hybridization, the filter was washed twice with 0.1× standard saline citrate/0.1% sodium dodecyl sulfate at 70°C (high stringency) or 55°C (low stringency) for 15 min. The DIG-labeled probe was detected with sheep anti-DIG antibody conjugated with alkaline phosphatase. The antibody detection reaction was performed using an enhanced chemiluminescent detection system (Boehringer Mannheim, Mannheim, Germany). The DIG-labeled 351 probe was made from exon 4 of *TIAP/m-survivin* cDNA as a template by polymerase chain reaction (PCR) using primers (5′-ACG CAT CCC AGC TTT TCC AG-3′ and 5′-TAT TCC GTT ACC CCG TGG TAG G-3′).

Isolation of genomic DNA clones homologous to the TIAP/m-survivin gene. Murine genomic DNA clones homologous to the *TIAP/m-survivin* gene were isolated from a 129/Sv genomic library using the murine TIAP/m-survivin probe (351 probe). A 17 kilo base pair (kb) fragment (G-3511) was obtained. A 5.5-kb *Xba*I fragment derived from G-3511 was subcloned into the *Xba*I site of pGEM-7Zf(+) (Promega, Madison, WI). Both strands were sequenced by an automatic DNA Sequencer (ALF Express, Pharmacia, Piscataway, NJ) with vector-specific primers.

Chromosomal localization of the TIAP-2 gene. The murine chromosomal localization of TIAP-2 was determined by radiation hybrid mapping. Radiation hybrid mapping was performed using the T31 mouse/hamster radiation hybrid panel (Research Genetics, Cat. RH0402) with two independent sets of primers: TIAP-2, 5′-1 (5′-GAG CCA TCA CTA GAG AAT GGT TGA C-3′) and TIAP-2, 3′-1 (5′-TGA AAA CTG GTG ACT CTG GTG GAG-3′); TIAP-2, 5′-2 (5′-TGC TGA GCC TTT GCT GAG ATA AC-3′) and TIAP-2, 3′-2 (5′-CCC ACT TTT CAA ATC CTC ATC CC-3′). The results obtained with these two sets of primers were consistent and were sent to the Jackson Laboratory RH database site for mapping of the gene relative to the RH map of the mouse genome.

Reverse-transcribed polymerase chain reaction (RT-PCR) analysis. Total RNAs were extracted from various organs from mice and NIH3T3 cells using the Trizol reagent (GIBCO/BRL, Rockville, MD). RNAs were reverse-transcribed using the TaKaRa RNA PCR kit (TaKaRa, Co. Ltd., Japan) and oligo(dT) M4 adaptor primer, in a final volume of 20 μl, and 1 μl of cDNAs was used for PCR. After an initial 5-min incubation at 94°C, the 35 cycles of PCR reactions were carried out using the following conditions: TIAP-2 cDNA; denaturation at 94°C for 0.5 min, annealing at 50°C for 0.5 min, and polymerization at 72°C for 2 min. G3PDH cDNA; denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and polymerization at 72°C for 1 min. PCR primers for the cDNA amplification were as follows: TIAP-2 primers; 5′-GAA AGC ACT CCC CTG GCT GCG CCT TC-3′ and M13 primer M4 (adaptor primer), and G3PDH primers; 5′-TGA AGG TCG GTG TGA ACG GAT TTG GC-3′ and 5′-CAT GTA GGC CAT GAG GTC CAC CAC-3′ (26). The PCR products were separated on a 1.5% agarose gel and detected by Southern blot with the 351 probe at low stringency.

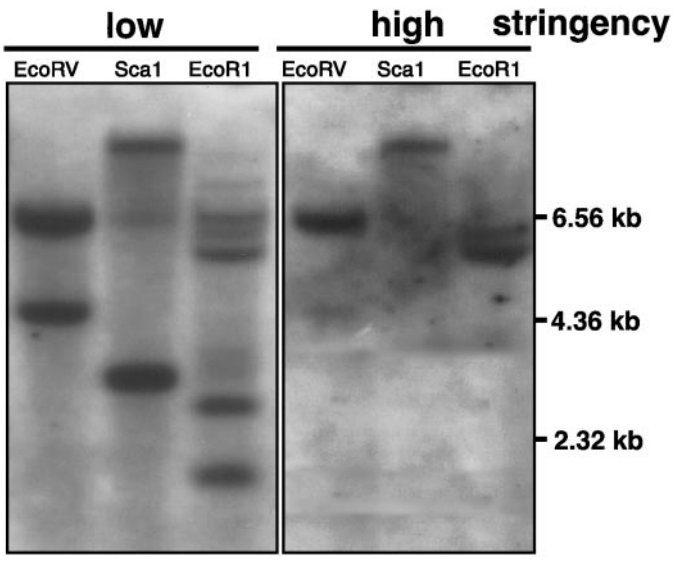

FIG. 1. Genomic Southern blot of the *TIAP/m-survivin* gene. Murine genomic DNA was digested with *Eco*RV, *Sca*1, or *Eco*R1, and genomic Southern blot was performed with the 351 probe. The filter was washed at high or low stringency.

Cell culture and cell cycle analysis. NIH3T3 (mouse fibroblast) cells were maintained in Dulbecco's modified Eagle medium (DMEM) (GIBCO/BRL, Rockville, MD) with 10% fetal calf serum (Bioserum, Victoria, Australia), 100 U/ml penicillin and 100 μg/ml Streptomycin at 37°C at 5% CO_2/95% air. For cell cycle analysis, NIH3T3 cells were cultured in 0.1% fetal calf serum for 48 h and released to re-enter the cell cycle by addition of serum (21). One million NIH3T3 cells were incubated in 1 ml of Krishan's reagent (0.05 mg/ml propidium iodide, 0.1% Na citrate, 0.02 mg/ml Ribonuclease A, 0.3% NP-40, pH 8.3) on ice for 30 min. Fluorescence from propidium iodide-nuclear DNA complexes was analyzed using a FACSCalibur (Becton Dickinson, San Jose, CA). Proportions of cells in G1, S and G2/M phase of the cell cycle were analyzed using the ModFit LT software (Verity Software House, Inc., Topsham, ME).

RESULTS AND DISCUSSION

Genomic cloning of a DNA region homologous to the TIAP/m-survivin gene. To examine the presence of TIAP/m-survivin-related gene(s), we performed genomic Southern blot with a probe (351 probe) carrying the 3′UT of *TIAP/m-survivin* cDNA at low stringency. When mouse genomic DNA was digested with *Eco*RV, the 351 probe detected 5.5- and 4.0-kb bands at low stringency but a 5.5 kb band at high stringency (Fig. 1). When mouse genomic DNA was digested with *Sca*1 or *Eco*R1, an extra 3.2 kb band or two extra 2.8 and 1.8 kb bands were detected at low stringency, respectively. These results suggested the presence of a novel gene belong to the *TIAP/m-survivin* gene family.

Mouse genomic library was screened with the 351 probe at low stringency. A clone (G-3511) was selected within several clones. Since the 5-kb *Xba*1 fragment of

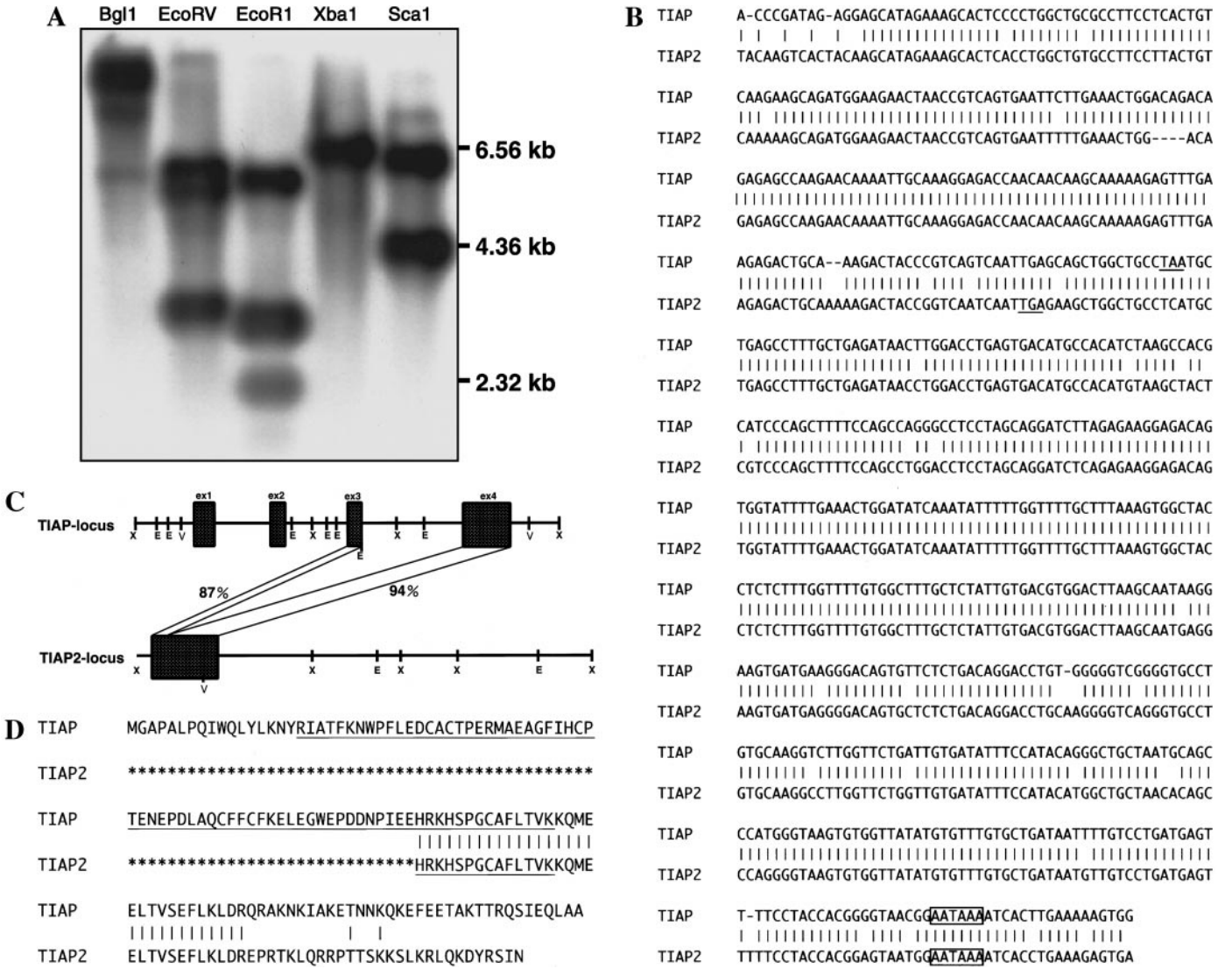

FIG. 2. Genomic organization of the *TIAP-2* gene. (A) A genomic DNA clone (G-3511) was digested with various enzymes and the homologous region was identified by Southern blot with the 351 probe at low stringency. (B) A part of DNA sequence of the 5-kb *Xba*I fragment of the *TIAP-2* gene and its homologous DNA sequence of the *TIAP/m-survivin* gene. The underlined sequences are stop codons and the boxed sequences are polyA additional sequences. (C) Genomic maps of the G-3511 clone and the *TIAP/m-survivin* gene. Shaded boxes indicate exons of those genes. V, *Eco*RV; X, *Xba*1; E, *Eco*R1. (D) The predicted amino acid sequence of TIAP-2 and the amino acid sequence of the homologous region of TIAP/m-survivin. The underlined sequences are the BIR domain.

G-3511 contained the DNA region homologous to the *TIAP/m-survivin* cDNA determined by Southern blot (Fig. 2A), the DNA fragment was subcloned, sequenced and compared with that of the *TIAP/m-survivin* gene. Since a part of the DNA sequence was quite similar to the 3′ side of the BIR domain in exon 3 and the 3′ UT in exon 4 of the *TIAP/m-survivin* cDNA and contained a putative TGA stop codon and the polyA additional signal sequence (Fig. 2B), the DNA region was tentatively named *TIAP-2*. Homology of *TIAP-2* to exon 3 and 4 of *TIAP/m-survivin* cDNA was 87% and 94%, respectively (Fig. 2C). The expected amino acid sequence of TIAP-2 contained a part of the BIR domain, and homology between TIAP-2 and TIAP/m-survivin was 55%, especially a part of the BIR domain (14 amino acids) was 100% identical between them (Fig. 2D).

The localization of *TIAP-2* on murine chromosomes was determined by radiation hybrid mapping. The *TIAP-2* is located between D9Mit223 and D9Mit88 on the chromosome 9 (D9Mit60-(12.7 cR)-D9Mit61-(79.2 cR)-D9Mit223-(1.0 cR)-TIAP-2-(28.4 cR)-D9Mit88-(3.8 cR)-D9Mit89-(34.8 cR)-D9Mit90; lod score >3.0). Since the *TIAP/m-survivin* gene is on the telomere of chromosome 11 (27), the *TIAP-2* is distinct from the *TIAP/m-survivin* gene.

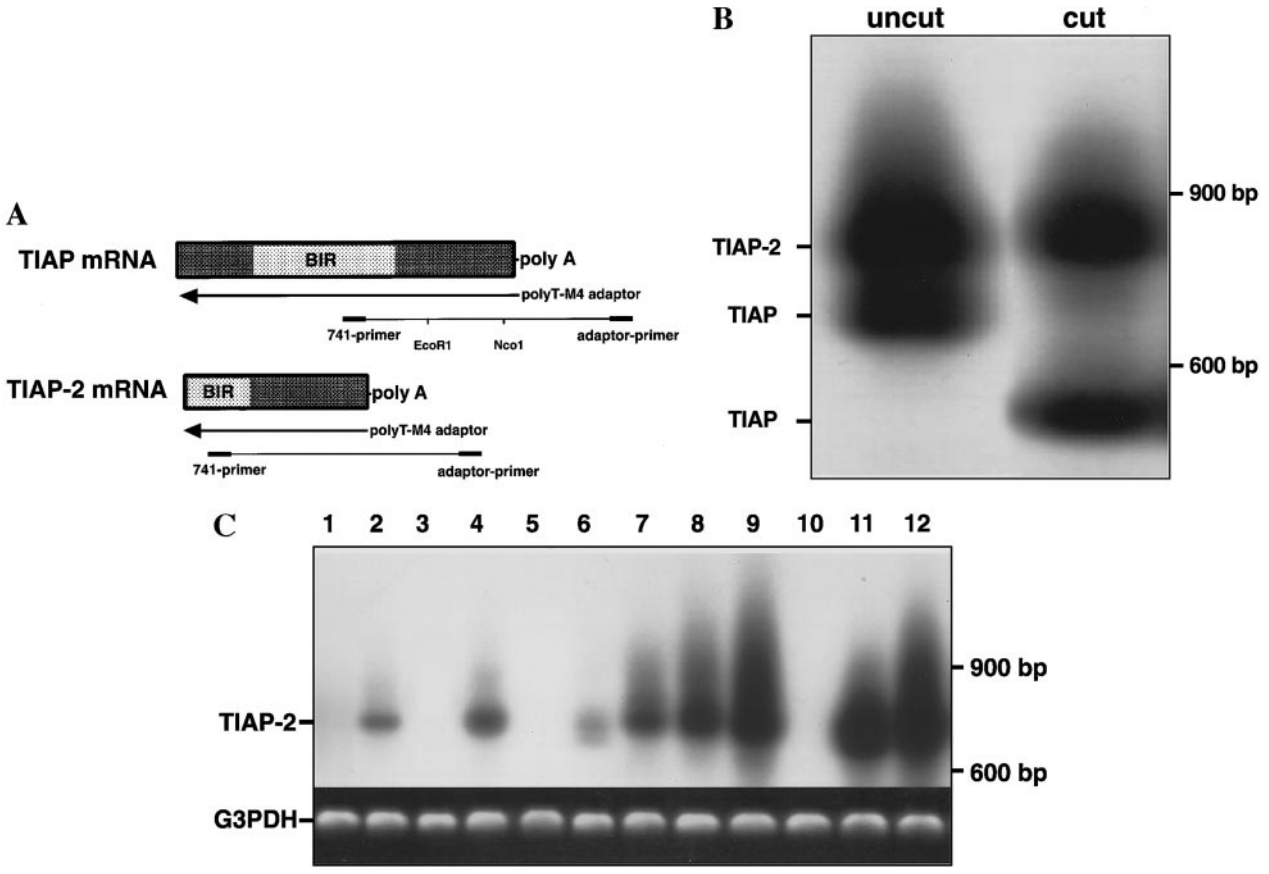

FIG. 3. Detection of *TIAP-2* mRNA by RT-PCR. (A) The method of RT-PCR to detect *TIAP-2* mRNA. (B) Expression of *TIAP-2* mRNA in bone marrow cells was detected by the RT-PCR (cut); PCR products were digested with *Eco*R1 and *Nco*1. (C) Expression of *TIAP-2* mRNA in various tissues. Expression of *TIAP-2* mRNA in various tissues from C57BL/6 mice at 8 weeks of age was detected by the RT-PCR. PCR products were digested with *Eco*R1 and *Nco*1. Lane 1, brain; 2, heart; 3, lung; 4, liver; 5, pancreas; 6, kidney; 7, intestine; 8, ovary; 9, testis; 10, muscle; 11, uterus; 12, thymus.

Expression of TIAP-2 mRNA. Expression of *TIAP-2* mRNA was analyzed in total RNAs from various tissues by Northern blot. However, there is no specific probe that can distinguish *TIAP-2* mRNA from *TIAP/m-survivin* mRNA. Thus, we tried to detect *TIAP-2* mRNA by RT-PCR with polyT-linker as a primer for the preparation of cDNA. Since *TIAP/m-survivin* but not *TIAP-2* has an *Eco*R1 and a *Nco*1 site in the cDNA region (Fig. 3A), RT-PCR products were digested with those enzymes to distinguish between them. TIAP-2 cDNA in those digested products was detected by Southern blot with the 351 probe. As shown in Fig. 3B, two bands were detected in total RNA from bone marrow cells. Since the lower band but not the upper band was digested with the enzymes, the upper band was expected as *TIAP-2* mRNA. Thus, expression of *TIAP-2* mRNA was examined in various tissues by this RT-PCR method. Figure 3C shows that *TIAP-2* mRNA was expressed in various tissues except brain, lung, pancreas, and muscle.

TIAP/m-survivin is expressed in S/G2/M phase of the cell cycle in a cycle-regulated manner (22) and associates with microtubules of the mitotic spindle in a specific and saturable reaction that is regulated by microtubule dynamics (24). Disruption of survivin-microtubule interactions by inhibiting expression of endogenous survivin with antisense DNA results in loss of survivin's anti-apoptosis function and increased caspase-3 activity, a mechanism involved in cell death, during mitosis (17). Thus, the relation of TIAP-2 expression with cell cycle was examined. NIH3T3 cells were starved of serum for 48 h and released to enter the cell cycle by re-stimulation with serum. The percentage of cells in S/G2/M phase of the cell cycle reached a peak (approximately 90%) at 16 h after re-stimulation, and most cells had re-entered the G1 phase 24 h after re-stimulation. Expression of *TIAP-2* mRNA in those cells was examined by RT-PCR analysis (Fig. 4). The level of *TIAP/m-survivin* mRNA was up-regulated at 12 h, reached a peak at 19 h and was slightly reduced at 24 h after restimulation (22). However, *TIAP-2* mRNA was constantly detected in those cells at any time examined. These results suggest that a role of TIAP-2 is distinct from that of TIAP/m-survivin.

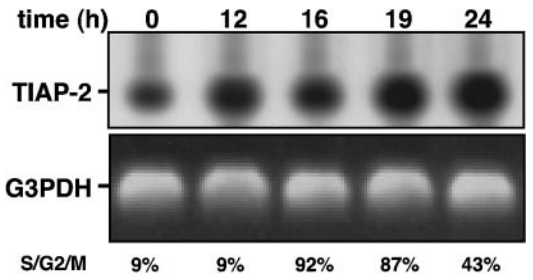

FIG. 4. Expression of *TIAP-2* mRNA in synchronized NIH3T3 cells. Expression of *TIAP-2* mRNA was detected in synchronized NIH3T3 cells by the RT-PCR. PCR products were digested with *Eco*R1 and *Nco*1. The percentage of cells in S/G2/M phase of the cell cycle detected by cell cycle analysis was described.

Recent reports indicate that there are several splice variants of *TIAP/m-survivin* gene (28, 29). Those variants are also detected in the *Survivin* gene of humans. Those sequence alterations cause marked changes in the structure of the corresponding proteins, including structural modifications of the BIR domain, indicating that function of those variants is distinct each other. Furthermore, tissue expression pattern of those variants is different. We have identified a part of the *TIAP-2* gene which tissue expression pattern is distinct from that of *TIAP/m-survivin*. Thus, a complex regulatory balance among the different isoforms of TIAP/m-survivin and TIAP-2 may determine the response to proapoptotic and proliferative stimuli.

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