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Molecular Cloning of a Human cDNA Encoding a Novel Protein, DAD1, Whose Defect Causes Apoptotic Cell Death in Hamster BHK21 Cells

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The tsBN7 cell line, one of the mutant lines temperature sensitive for growth which have been isolated from the BHK21 cell line, was found to die by apoptosis following a shift to the nonpermissive temperature. The induced apoptosis was inhibited by a protein synthesis inhibitor, cycloheximide, but not by the *bcl-2*-encoded protein. By DNA-mediated gene transfer, we cloned a cDNA that complements the tsBN7 mutation. It encodes a novel hydrophobic protein, designated DAD1, which is well conserved (100% identical amino acids between humans and hamsters). By comparing the base sequences of the parental BHK21 and tsBN7 DAD1 cDNAs, we found that the DAD1-encoding gene is mutated in tsBN7 cells. The DAD1 protein disappeared in tsBN7 cells following a shift to the nonpermissive temperature, suggesting that loss of the DAD1 protein triggers apoptosis.

Programmed cell death is a mechanism for eliminating unwanted cells which is essential for the social control of cell proliferation (15). In the nematode *Caenorhabditis elegans*, genes involved in programmed cell death have been identified (3). The *ced-3* and *ced-4* genes are essential for programmed cell death, while the *ced-9* gene acts as a brake against programmed cell death. If the function of *ced-9* is inactivated by mutation, cells that should normally survive instead die, by a pathway which depends upon the function of *ced-3* and *ced-4*.

As in *C. elegans*, in cultured animal cells, the existence of a specific pathway toward apoptosis has been suggested (19). The mammalian gene *bcl-2* appears to resemble *C. elegans* gene *ced-9*. Overexpression of the *bcl-2*-encoded protein protects lymphocytes from apoptosis and also protects *C. elegans* from cell death mediated by CED-3 and CED-4 (21). However, the function of *bcl-2* is not universal. Cytotoxic T-cell killing, which has been suggested to have many features in common with apoptosis, was not suppressed by *bcl-2* (20). The cellular pathways leading to apoptosis appear to be intricate. The molecular mechanisms of programmed cell death by apoptosis are far from being clarified.

To identify the genes involved in apoptosis, we searched through a collection of temperature-sensitive (ts) mammalian cells. This method may be able to identify genes like *ced-9*, loss of whose function induces apoptosis. The collection of mutants which are ts for cell growth and were used in this study was isolated from the BHK21 cell line derived from golden hamsters, and these mutants have been classified into complementation groups (9, 11). Cell line tsBN7, which is one of these mutants, rapidly rounds up and comes off the dish at the nonpermissive temperature. We therefore chose this mutant as a first candidate for ts mutants entering apoptosis following a temperature shift. By using tsBN7 cells as a recipient of gene transfer, we cloned a human cDNA that encodes a novel protein designated DAD1. DAD1 cDNA complemented the tsBN7 mutation. We showed that the hamster DAD1-encoding gene, which produces the same amino acid sequence as the human homolog, is mutated in tsBN7 cells. Because of the mutation, the DAD1 protein disappeared in tsBN7 cells following a shift to the nonpermissive temperature, suggesting that loss of DAD1 function causes apoptosis.

MATERIALS AND METHODS

Cell lines and cell culture. The tsBN7 cell line is a ts mutant derived from the BHK21 cell line (9). The HeLa cell line is derived from human cancer of the uterine cervix.

All cell lines were cultured in Dulbecco modified Eagle medium supplemented with 10% calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere containing 10% CO_2.

Except for ts mutants, cell lines were cultured at 37.5°C. tsBN7 cells were maintained at the permissive temperature, 33.5°C, and ts^+ cells were selected at the nonpermissive temperature, 39.5°C.

Macromolecular synthesis. Synthesis of DNA, RNA, and protein at both 33.5 and 39.5°C was measured by incorporation of radioactive precursors into trichloroacetic acid-insoluble materials as described by Nishimoto et al. (10).

Electron microscopy. Cells were collected and washed twice with an isotonic buffer, Tris-buffered saline containing 136.8 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 25 mM Tris-HCl (pH 7.4). The cell pellets were prefixed for 30 min with 3% glutaraldehyde in 0.1 M cacodylate buffer, postfixed for 30 min with 1% osmium tetroxide in 0.1 M cacodylate buffer, and stained en bloc with 2% uranyl acetate for 30 min. After dehydration in a graded series of ethanol solutions, the samples were embedded in Epon. Thin sections were prepared on an ultramicrotome (Reihert-Jung Optische

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Werke AG, Vienna, Austria), stained with uranyl acetate and lead citrate, and then examined with a 2000-EX electron microscope (JEOL, Tokyo, Japan) operated at 100 kV.

Detection of DNA fragmentation. Total cellular DNA was isolated by phenol extraction, incubated for 30 min in the presence of RNase (10 μg/ml) as previously described (6), and electrophoresed in a 2.0% agarose gel in TAE buffer (0.04 M Tris acetate, 1 mM EDTA). The gel was stained with ethidium bromide.

Transformation. The cDNA library derived from human Raji cells constructed in the pcDEB vector (5) was a gift from H. Hayakawa. The *bcl-2* cDNA inserted into pBC140 was a gift from Thomas von Ruden. High-molecular-weight cellular DNA was extracted as previously described (23). pSV2-neo was cotransfected as a marker of transfection.

DNA was transfected into cultures of the tsBN7 cell line (2×10^5 cells per 100-mm-diameter dish) with the calcium phosphate precipitation method as modified by Chen and Okayama (2). Transfected cells were incubated at 33.5°C for 17 h, washed twice with Dulbecco modified Eagle medium without serum, and cultured at 33.5°C with or without either hygromycin B (300 μg/ml) or G418 (400 μg/ml). When tiny colonies appeared at 33.5°C in the presence of selective drugs, cells were fed the fresh medium without drugs and then cultured at 39.5°C. About 2 weeks later, the colonies were counted.

Isolation of cDNA clones. (i) Human DAD1 cDNA. One microgram of genomic DNA from ts$^+$ transformants was amplified by using synthetic oligonucleotides and the following program: 94°C for 1 min, 65°C for 2 min, and then 72°C for 3 min for 30 cycles. Amplified DNA fragments were purified from the agarose gel by using SUPREC-01 (Takara, Kyoto, Japan), and these were subcloned either into pUC19 for sequencing or into pcDL-SRα296 (17) for complementation assay.

(ii) BHK21 DAD1 cDNA. A cDNA library of BHK21 cells constructed in the λgt10 vector was screened by using human DAD1 cDNA as a probe as described previously (13).

(iii) tsBN7 DAD1 cDNA. Ten micrograms of total RNA extracted from tsBN7 cells was reverse transcribed to prepare the first-strand cDNA by using primer 2 (5′-TTCTGC CAACCTCCAACTCCG-3′) and the Amersham cDNA synthesis kit as recommended by the supplier. The cDNA was then amplified by using primer 1 (5′-GTCCGAAGGTCCC GCGCTCGTC-3′) and primer 2 with *Taq* polymerase (the underlined sequence is derived from the genomic sequence). The polymerase chain reaction (PCR) was carried out as follows, 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min for 30 cycles.

(iv) *Xenopus* DAD1 cDNA. A *Xenopus laevis* cDNA library constructed in the λgt10 vector (16) was screened by using hamster DAD1 cDNA as a probe.

Northern (RNA) blot analysis. A multiple-tissue Northern blot filter was purchased from Clontech (Palo Alto, Calif.). Each lane contained 2 μg of poly(A)$^+$ mRNA extracted from multiple human organs. The filter was prehybridized with a buffer containing 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 10× Denhardt's solution, 100 μg of salmon sperm DNA per ml, 50% formamide, and 2% sodium dodecyl sulfate (SDS) for 3 h at 42°C and then hybridized with a ^{32}P-labelled cDNA probe at 42°C overnight. After hybridization, the filter was washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at room temperature, for 15 min each time, and twice with 1× SSC–0.1% SDS at 42°C, for 15 min each time. The washed filter was exposed and analyzed with a BAS2000 Bio Image Analyzer (Fuji).

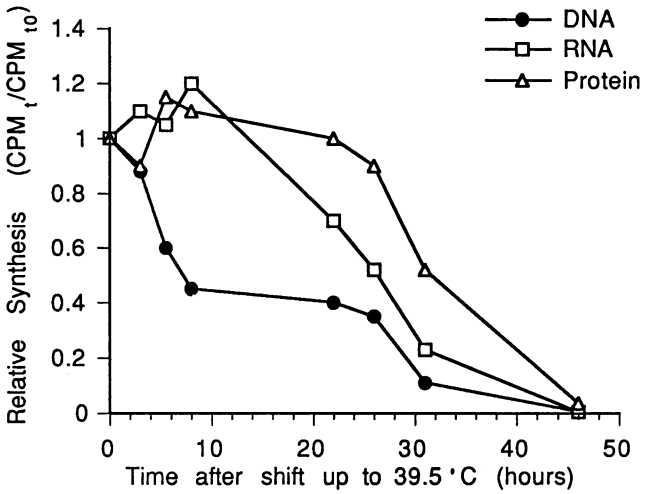

FIG. 1. Macromolecular synthesis of tsBN7 cells. Cells were seeded at 10^4/35-mm-diameter dish, incubated at 33.5°C for 2 days, and then shifted to 39.5°C. At the times indicated, the cells were labeled for 1 h with [^{3}H]thymidine (DNA), [^{3}H]uridine (RNA), or a ^{3}H-labelled amino acid mixture. The ordinate gives the ratio of radioactivity incorporated at time t to the radioactivity incorporated at the time of the temperature shift up (t_0).

DNA sequencing. DNA fragments were subcloned into multiple cloning sites of the pUC19 vector and sequenced by the dideoxy-chain termination method in both directions. The nucleotide sequences determined were compiled and analyzed with the DNASIS programs (Hitachi Software Engineering Co. Ltd.).

Preparation of antipeptide antibody. According to the sequence of human-hamster DAD1 cDNA, a peptide with the sequence NPQNKADFQGISPER was synthesized. Cysteine was added to the carboxy terminus to couple the carrier protein keyhole limpet hemocyanin. The peptide coupled to the carrier protein was used to immunize rabbits.

Immunoblotting. Cells were lysed in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 10 mM 2-mercaptoethanol, 3% (wt/vol) SDS, and 20% glycerol. Cellular proteins were electrophoresed in an SDS–15% polyacrylamide slab gel and analyzed by immunoblotting as described previously (12).

Nucleotide and amino acid sequence accession numbers. The GenBank-EMBL accession numbers for the human, hamster, and *Xenopus* DAD1 nucleotide and amino acid sequences are D15057, D15058, and D15059, respectively.

RESULTS

Apoptosis is induced in tsBN7 cells by a temperature shift. The tsBN7 cell line, one of the ts mutant lines isolated by Nishimoto and Basilico (9), has a single recessive mutation. It normally grows at 33.5°C but not at 39.5°C. After a shift to 39.5°C, DNA synthesis decreased to less than half of the normal level in 10 h while protein synthesis was maintained for 30 h (Fig. 1). Between 30 and 35 h, cells rounded up and detached from the substrate (Fig. 2a).

The detached cells had condensed chromatin at the periphery of the nucleus and blebbed and vacuolized cytoplasm and lacked microvilli; however, the structure of cytoplasmic organelles appeared to be preserved (Fig. 3A).

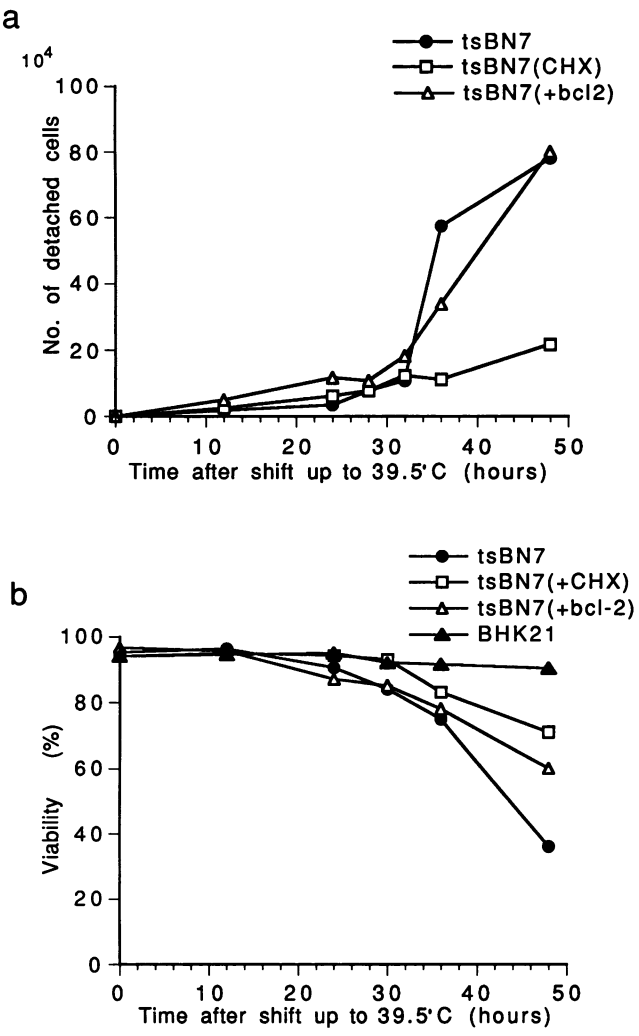

FIG. 2. Kinetics of cell detachment and cell death. (a) Cell detachment tsBN7 cells (2×10^5) and a *bcl-2* transformant (+bcl2) were plated on 100-mm-diameter dishes and incubated at 33.5°C for 48 h. The cells were given fresh medium containing 10% calf serum or 10% calf serum with cycloheximide (10 μg/ml) (CHX) and incubated at 39.5°C. At the times indicated, floating cells were counted with a Coulter Counter. (b) Cell viability. Cells cultured as described above were collected at the indicated times after a shift to 39.5°C, and their viability was determined by trypan blue dye exclusion.

These are all characteristic features of apoptosis (22, 25). Consistent with these findings, tsBN7 cells lost their viability with the same kinetics as cell detachment (Fig. 2b).

After incubation at 39.5°C for 36 h, the cellular DNA was degraded to oligonucleosomal fragments as previously reported for apoptosis (3) (Fig. 3B). At 39.5°C, wild-type BHK21 cells showed neither the morphological change (Fig. 3A) nor the fragmentation of DNA (data not shown), indicating that apoptosis in tsBN7 cells cultured at 39.5°C was not a consequence of heat shock due to culturing at a high temperature. These results indicated that the tsBN7 cell line contains a ts mutation that causes cells to undergo apoptosis following a shift to 39.5°C.

Preliminary characterization of apoptosis caused by the tsBN7 mutation. Since the *bcl-2* gene has been reported to prevent apoptosis in some cases (reviewed in references 15 and 19), we transfected tsBN7 cells with *bcl-2* cDNA inserted into expression vector pBC140, which has the Neo^r-encoding gene (1). The Neo^r transformants did not grow at 39.5°C (Table 1). They died at 39.5°C with little, if any, delay compared with nontransfected tsBN7 cells (Fig. 2). Cellular DNA of Neo^r transformants was fragmented at 39.5°C, despite overexpression of the *bcl-2*-encoded protein (Fig. 3C). Thus, the *bcl-2* gene does not seem to block apoptosis caused by the tsBN7 mutation.

On the other hand, cycloheximide prevented both cell detachment and fragmentation of DNA in tsBN7 cells cultured at 39.5°C (Fig. 2 and 3B), suggesting that apoptosis caused by the tsBN7 mutation depends upon protein synthesis.

The human gene that complements the tsBN7 mutation. To identify the gene mutated in the tsBN7 cell line, a human cDNA library constructed in an expression vector, pcDEB, containing the hygromycin B resistance (HB^r)-encoding gene (5) was transfected into tsBN7 cells by the method of Chen and Okayama (2). Among 1.5×10^4 HB^r colonies, 14 ts$^+$ colonies were isolated (Table 1). Total cellular DNA extracted from these primary ts$^+$ transformants was again transfected into tsBN7 cells; that is, secondary transformation was carried out. The frequency of ts$^+$ transformation was 0.1×10^{-5} to 0.6×10^{-5} through the primary and secondary transformations (Table 1).

From primary and secondary ts$^+$ transformants, transfected human cDNA fragments about 800 bp long were amplified and recovered by PCR as described in Materials and Methods, by using as primers the vector sequences flanking the cDNA insert (Fig. 4). The recovered cDNA was subcloned into a mammalian expression vector, pcDL-SRα296 (17), to examine its ability to complement the tsBN7 mutation. The isolated human cDNA efficiently transformed tsBN7 cells to the ts$^+$ phenotype (Table 1, human DAD1), indicating that the cloned cDNA is biologically active.

In the cloned cDNA fragment, we found an open reading frame of 339 bp that encodes a protein with a molecular mass of 12.5 kDa (Fig. 5). The nucleotide sequence upstream from the putative first ATG codon is GCAGTTATG, in accordance with Kozak's rule (CCA/GCCATG) (7). A polyadenylation signal (AATTAAA) was located 19 bp upstream from the poly(A)$^+$ tail.

No homologous proteins were found by using the programs in DNASIS. Thus, the cloned human cDNA encodes a novel protein. We designated the protein encoded by the cloned human cDNA DAD1 for defender against apoptotic cell death.

Presence of the putative DAD1 protein in human cells. By Northern analysis, a single abundant 0.8-kb band, the same length as the isolated DAD1 cDNA, was detected in human cellular RNA isolated from all of the organs so far examined (Fig. 6). Thus, the DAD1-encoding gene is expressed at a relatively high level all over the body. To prove that the putative DAD1 protein is present in human cells, an antibody to the synthetic DAD1 peptide (the position of the peptide is shown in Fig. 5) was prepared as described in Materials and Methods.

A lysate of exponentially growing HeLa cells was prepared and subjected to immunoblotting analysis with the antipeptide antibody. A protein of 12 kDa, the same size as that calculated on the basis of the amino acid sequence of the putative DAD1 protein, was recognized by the antibody, but not in the presence of a competitor (see Fig. 8A). Thus, the

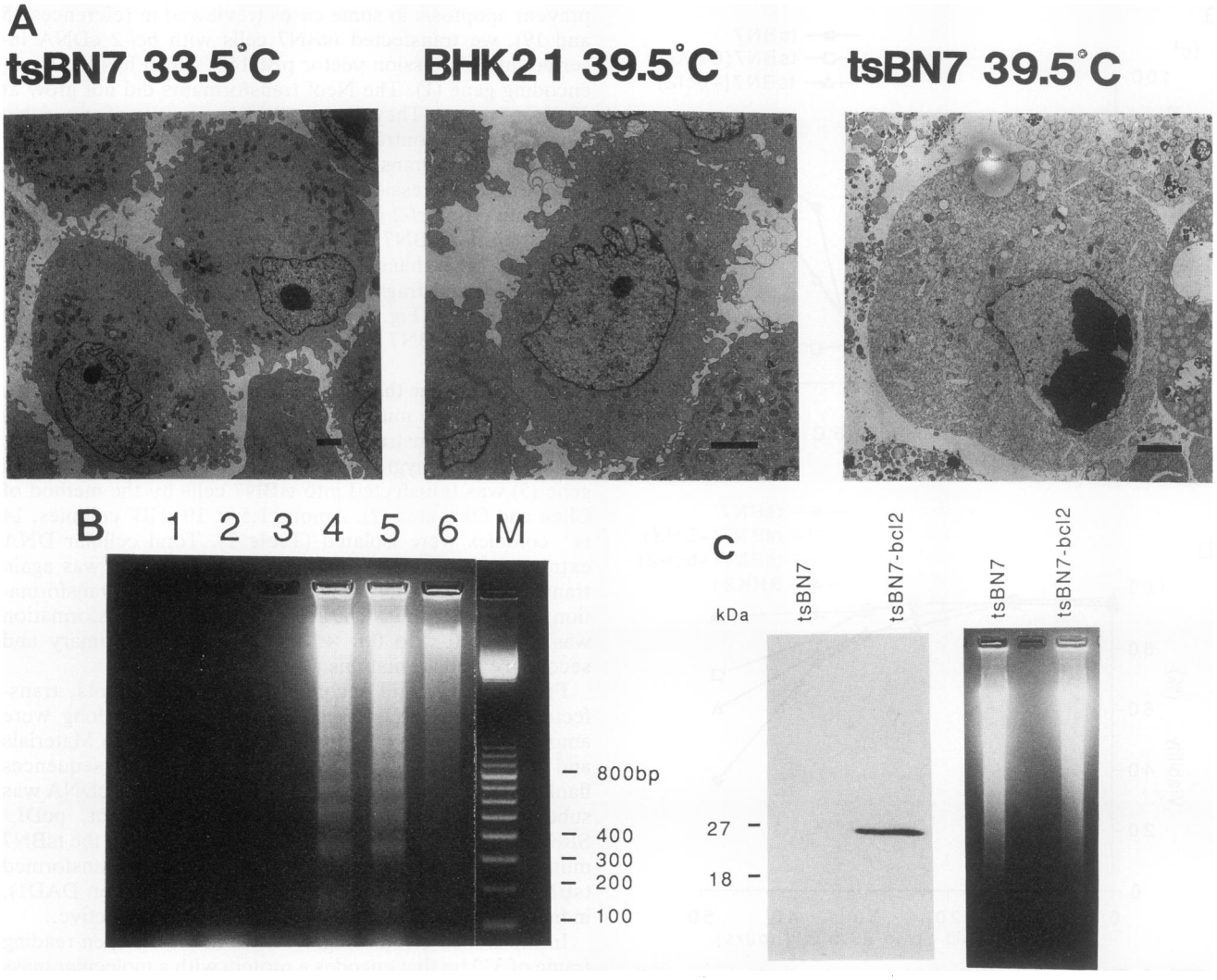

FIG. 3. Apoptosis is induced in tsBN7 cells following a shift to the nonpermissive temperature. (A) Electron micrographs of tsBN7 and BHK21 cells. Cells incubated as indicated at either 33.5 or 39.5°C for 48 h were collected and washed twice with the isotonic buffer. The cell pellets were processed to be examined by electron microscopy as described in Materials and Methods. (B) DNA fragmentation of tsBN7 cells cultured at 39.5°C. Total cellular DNA extracted from tsBN7 cells cultured at 39.5°C for 0 (lane 1), 12 (lane 2), 24 (lane 3), 36 (lane 4), or 48 (lane 5) h or in the presence of cycloheximide for 48 h (lane 6) was electrophoresed as described in Materials and Methods. (C) DNA fragmentation of tsBN7 cells was not prevented by *bcl-2* expression. Left panel: immunoblotting analysis of total cell lysates with anti-BCL-2 antibody. The antibody used was an anti-human BCL-2-specific monoclonal antibody (14). Right panel: fragmentation of total cellular DNA. Bars, 2 μm.

DAD1 protein predicted by the nucleotide sequence actually exists in human cells.

The amino acid sequence suggested that the DAD1 protein is highly hydrophobic, but the length of clustered hydrophobic residues does not seem to be enough to span the membrane (8) (Fig. 5B).

The DAD1-encoding gene is mutated in the tsBN7 cell line. To investigate the role of protein DAD1 in apoptosis induced by the tsBN7 mutation, it should be proved that the DAD1-encoding gene is, in fact, mutated in this cell line. To do this, we cloned both parental BHK21 and tsBN7 DAD1 cDNAs.

A hamster cDNA library constructed in the λgt10 vector was screened by using human DAD1 cDNA as a probe. After screening 80,000 clones, we obtained 3 positive clones of about 700 bp. The cloned hamster cDNA had an open reading frame of 339 bp. The sequence upstream of the putative ATG codon (CGCTATG) conformed to Kozak's rule (7).

The tsBN7 DAD1 cDNA from tsBN7 cells was then cloned by a reverse-transcribed PCR with two oligonucleotides: primers 1 and 2. These primers were prepared on the basis of the base sequence of the untranslated region of the BHK DAD1 cDNA, as shown in Fig. 5. Three independently amplified clones were sequenced. Comparison of the nucleotide sequences of amplified tsBN7 cDNAs with that of the BHK21 cDNA revealed that the first base of the 38th codon, guanine, was changed to adenine in tsBN7 cDNA (Fig. 7b). By this base change, glycine was converted to arginine in the tsBN7 DAD1 protein (Fig. 7c). No other base change was found in tsBN7 DAD1 cDNA.

Base change in the genomic DNA. The base change found in the cDNA was confirmed to exist in the genomic DNA. We

TABLE 1. Transformation of tsBN7 cells with the cDNA library and the cloned cDNAs[a]

Donor DNA	No. of colonies (10^5)		
	HB^r	ts$^+$	Neo^r
pcDEB + cDNA library[b]	200	0.19	
pcDEB vector only	200	<0.1	
Total cellular DNAs[c]			
7EB-19		0.1	
7EB-24		0.6	
tsBN7		<0.1	
pcDL-sRα–human DAD1[d]		97.5	ND
pcDL-sRα–BHK21 DAD1		69	87
pcDL-sRα–tsBN7 DAD1		0.2[e]	97
pBCbcl-2[f]		<0.1	124

[a] Transfections were carried out as described in Materials and Methods. For transfection with total cellular DNA, transformants were selected at 39.5°C without preselection with drugs.
[b] The pcDEB vector library was constructed by Hayakawa et al. (5).
[c] 7EB-19 and 7EB-24 are primary ts$^+$ transformants of tsBN7 cells transfected with the pcDEB cDNA library.
[d] pSV2-neo was cotransfected with cloned DAD1 cDNAs.
[e] Revertants (spontaneous frequency, 10^{-8} [9]).
[f] *bcl-2* cDNA was inserted into pBC140 (obtained from Thomas von Ruden, Research Institute of Molecular Pathology, Vienna, Austria), which has the cytomegalovirus promoter-enhancer and the Neo^r-encoding gene (1).

isolated the genomic DNA of the parental BHK DAD1 genome and determined the exon-intron junctions. The base sequence of isolated genomic DNA fragments indicated that the BHK DAD1 gene consists of three exons (Fig. 7a). The base change found in the cDNA was estimated to be located in the first exon. On the basis of this finding, we prepared primer 3, which corresponds to the 3′ region of the putative mutation site and is located in the first exon (the sequence of this primer shown in Fig. 5). DNA fragments of 150 bp, corresponding to the size calculated on the basis of the sequence of BHK DAD1 cDNA, were amplified from the genomic DNA of tsBN7 cells by PCR with primers 1 and 3. These were subcloned into vector pUC19 to be sequenced. The same base change as that found in the cDNA clone was detected in the genomic DNA fragments isolated independently (data not shown).

Effect of the base change on gene function. We confirmed that the base change we found is responsible for the biological activity of the DAD1-encoding gene. Both BHK21 and tsBN7 DAD1 cDNAs were subcloned into expression vector pcDL-SRα296 (17) and then transfected into tsBN7 cells as described in Materials and Methods. Unlike BHK DAD1 cDNA, tsBN7 DAD1 cDNA did not have the ability to convert tsBN7 cells to the ts$^+$ phenotype (Table 1), although the ORFs of both cDNAs were identical, except for the one amino acid at the 38th codon.

The DAD1 protein disappears in tsBN7 cells following a temperature shift. By using the algorithm of Robson, the structures of the wild-type BHK21 and tsBN7 DAD1 proteins were estimated. As a consequence of the point mutation at the 38th codon, an extra turn was predicted to be formed at the 40th residue of the tsBN7 DAD1 protein. As a result, the orientation of protein folding in the C-terminal portion becomes opposite to that of the wild-type protein (Fig. 7d). Previously, we found that such a structural change caused disappearance of the mutated protein following a temperature shift (12, 18). To investigate whether the same phenomenon happens in tsBN7 cells, the DAD1 protein of tsBN7 cells cultured at 39.5°C was analyzed by immunoblotting with the antibody to the DAD1 protein.

A series of exponentially growing cultures of BHK21 and tsBN7 cells was shifted to the nonpermissive temperature (Fig. 8B). At the times indicated in Fig. 8B, cells were collected and lysed as described in Materials and Methods. The DAD1 protein of tsBN7 cells, although its content is low even at 0 h compared with that of BHK21 cells, became undetectable after 6 h of incubation at 39.5°C, while the content of α-tubulin did not change. In BHK21 cells, such a disappearance of the DAD1 protein was not detected. Thus, in tsBN7 cells, the DAD1 protein disappeared following a temperature shift, indicating that apoptosis of tsBN7 cells was induced by loss of the DAD1 protein. Its lower content in tsBN7 cells growing at 33.5°C may reflect instability of the mutated protein.

The DAD1 protein is well conserved. The amino acid sequences of the DAD1 proteins estimated by the nucleotide sequences are completely identical between humans and hamsters (Fig. 5). To estimate the conservation of this protein, we further isolated the *Xenopus* homolog from the *Xenopus* cDNA library (16). The amino acid sequence of the *Xenopus* DAD1 protein is 91% identical (96% similar) to that of the human-hamster DAD1 protein (Fig. 9).

DISCUSSION

Our data show that tsBN7 cells have a ts mutation in the DAD1 gene. At 39.5°C, the nonpermissive temperature,

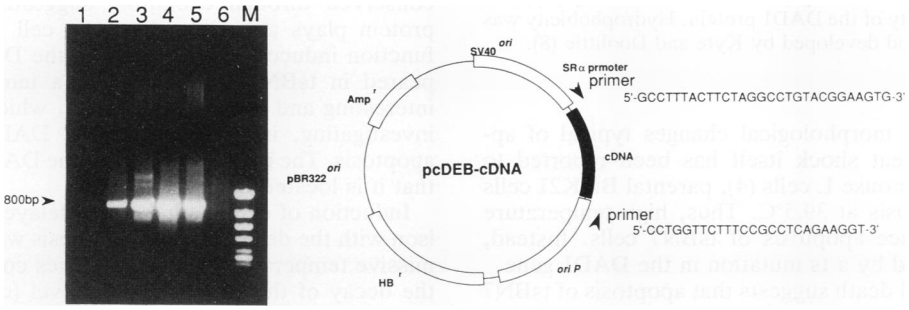

FIG. 4. Amplification of the cDNA conserved in ts$^+$ transformants of tsBN7 cells. One microgram each of genomic DNA extracted from primary ts$^+$ transformants 7EB19 (lane 1) and 7EB24 (lane 2); from secondary ts$^+$ transformants 7EB19-1 (lane 3), 7EB24-1 (lane 4), and 7EB24-3 (lane 5); and from BHK21 cells (lane 6) was amplified by PCR with the indicated primers as described in Materials and Methods. HB, hygromycin B; SV40, simian virus 40.

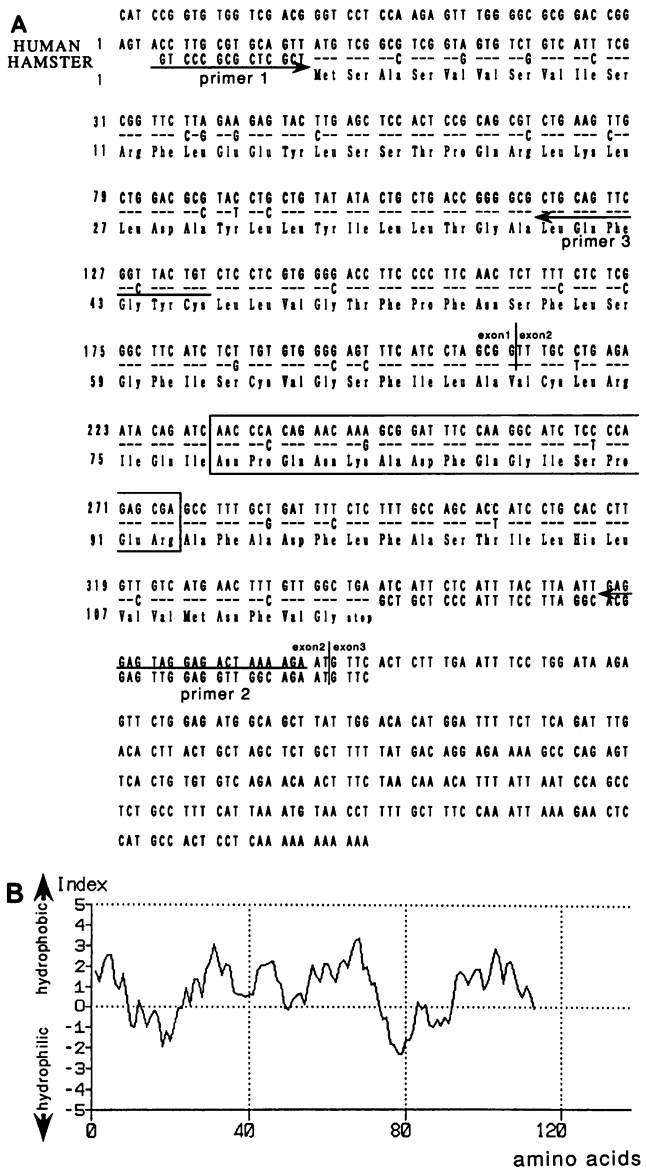

FIG. 5. Amino acid and nucleotide sequences of the human and hamster DAD1-encoding genes. (A) The nucleotide sequences of both the human and hamster DAD1 cDNAs are shown. Dashes in the hamster sequence indicate bases identical to those of human DAD1 cDNA. The arrows indicate the positions of primers used to determine the mutation site. The exon-intron junctions are also indicated. The box indicates the peptide used for antibody preparation. (B) Hydrophobicity of the DAD1 protein. Hydrophobicity was estimated by the method developed by Kyte and Doolittle (8).

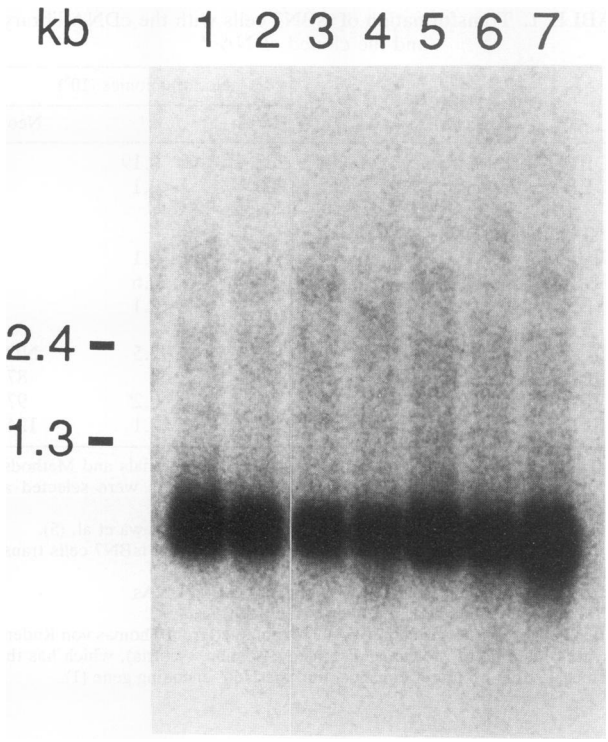

FIG. 6. Expression of the DAD1-encoding gene in multiple human organs. Two-microgram samples of poly(A)$^+$ RNA extracted from human organs were electrophoresed and subjected to Northern blot analysis as described in Materials and Methods. Lanes: 1, heart; 2, brain; 3, lung; 4, liver; 5, skeletal muscle; 6, kidney; 7, pancreas.

tsBN7 cells showed morphological changes typical of apoptosis. Although heat shock itself has been reported to induce apoptosis in mouse L cells (4), parental BHK21 cells did not enter apoptosis at 39.5°C. Thus, high temperature alone does not induce apoptosis of tsBN7 cells. Instead, apoptosis was caused by a ts mutation in the DAD1 gene.

The kinetics of cell death suggests that apoptosis of tsBN7 cells is unlike that induced by Fas antigen but rather belongs to the category of apoptosis caused by withdrawal of interleukin or nerve growth factor (3). The latter type of cell death is typically prevented by overexpression of the *bcl-2*-encoded protein (21). However, in the case of tsBN7 cells, apoptosis was not efficiently blocked by the human *bcl-2*-encoded protein. One possibility is that the DAD1 protein may function downstream of the *bcl-2*-encoded protein to prevent cell death. The DAD1 protein may inhibit production of a death protein(s), such as CED-3 or CED-4, since apoptosis of tsBN7 cells was prevented by cycloheximide. However, we have to say that there has been no evidence showing that the human *bcl-2* gene prevents apoptosis of hamster cells, although it protects *C. elegans* from cell death (21). It is, therefore, at least formally possible that human *bcl-2* does not function completely in hamsters.

The DAD1 protein is a novel protein. It seems to be well conserved through evolution, suggesting that the DAD1 protein plays an essential role in cell growth. Its loss of function induces apoptosis, since the DAD1 protein disappeared in tsBN7 cells following a temperature shift. An interesting and important question, which we are currently investigating, is how loss of the DAD1 protein induces apoptosis. The hydrophobicity of the DAD1 protein suggests that it is located in a membrane.

Induction of cell death is quite delayed (30 h) in comparison with the decay of DNA synthesis which, at the nonpermissive temperature, follows kinetics comparable to that of the decay of the DAD1 protein level (compare Fig. 1 and 8B). Thus, the apoptosis induced might be secondary to the defect in DNA synthesis at some critical level rather than to loss of direct interaction of DAD1 with cell death genes, analogous to the action of *ced-9* on *ced-3* and *ced-4* (3).

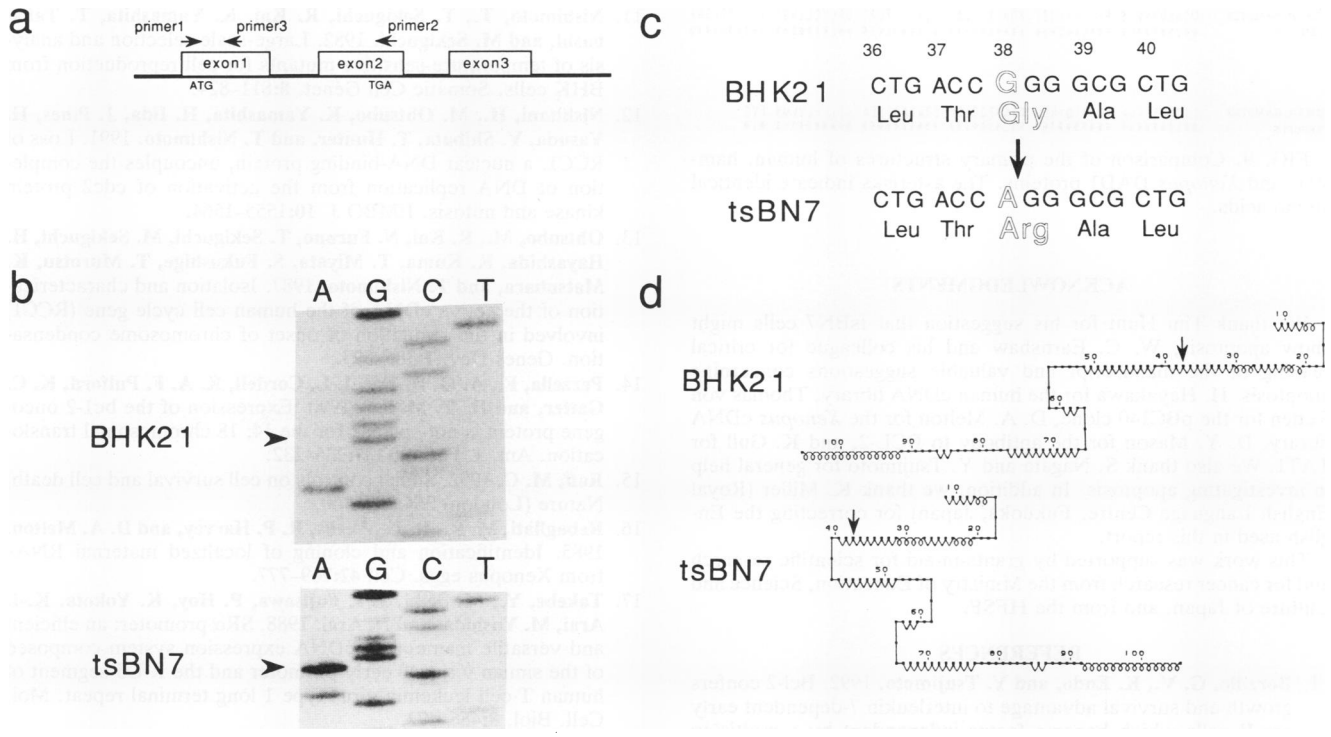

FIG. 7. The mutation site of the tsBN7 DAD1-encoding gene. (a) Structure of the DAD1 gene. The primers indicated were used for amplification of cDNA and genomic DNA. (b) Sequence ladders near the mutation sites in the BHK21 and tsBN7 DAD1 cDNAs. (c) Amino acid change predicted by the base change found. (d) Structural change caused by the mutation. The amino acid sequences of BHK21 DAD1 and tsBN7 DAD1 were analyzed by using the algorithm of Robson programmed in DNASIS (Hitachi Software Engineering). The arrows indicate the positions of the mutation sites.

BHK21 cells, however, did not enter apoptosis when DNA replication was prevented by a inhibitor of DNA synthesis, such as aphidicolin or hydroxyurea. Furthermore, another ts mutant of BHK21 cells, tsBN250, in which DNA replication is rapidly inhibited at 39.5°C (11) did not show fragmentation of DNA (unpublished data) at 39.5°C. Inhibition of DNA replication itself, therefore, does not cause apoptosis in BHK21 cells.

In *C. elegans*, two groups of genes have been shown to be involved with control of programmed cell death. These include genes required to kill cells, such as *ced-3* and *ced-4*, and genes that prevent cell killing, such as *ced-9* (3). ts defects in the second type of genes may result in cell death at the nonpermissive temperature, although ts mutants grow normally at the permissive temperature. Thus, ts mutant forms of genes that negatively regulate apoptosis could be identified among mutants ts for cell growth. The tsBN7 cell line is one such mutant. Our results open a new approach for the systematic investigation of negative regulators of apoptosis at the molecular level.

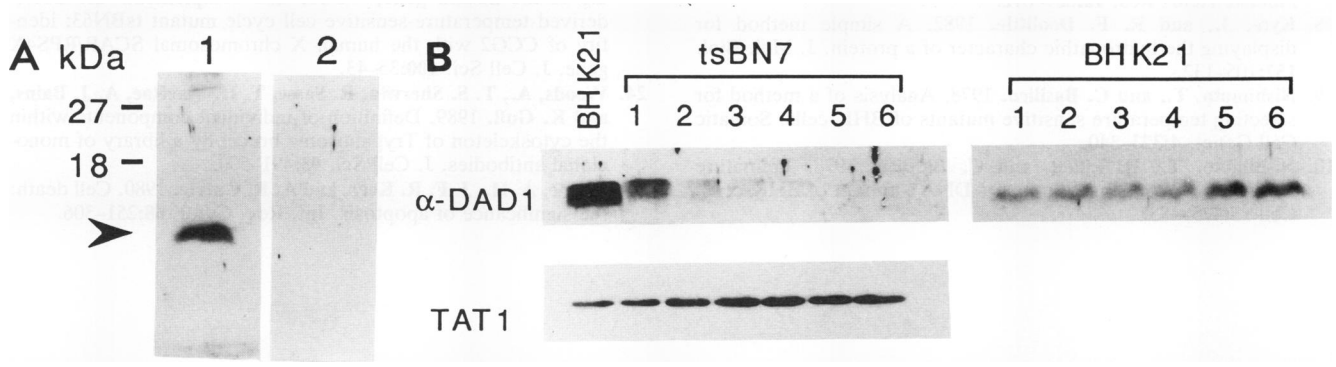

FIG. 8. Presence of the DAD1 protein in HeLa, BHK, and tsBN7 cells. (A) DAD1 protein detected in HeLa cells. Total cellular protein (30 μg) extracted from HeLa cells was subjected to SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with the anti-DAD1 peptide antibody. Lanes: 1, anti-DAD1 peptide antibody; 2, anti-DAD1 peptide antibody in the presence of 100 μg of the DAD1 peptide as a competitor. The arrowhead indicates the position of DAD1. (B) Disappearance of the DAD1 protein in tsBN7 cells upon temperature shift. Total cellular protein (30 μg) extracted from tsBN7 and BHK21 cells incubated at 39.5°C for 0 (lane 1), 6 (lane 2), 12 (lane 3), 24 (lane 4), 36 (lane 5), or 48 (lane 6) h was electrophoresed and analyzed by immunoblotting with the anti-DAD1 or the TAT1 (anti-α-tubulin) antibody (24). Lane BHK21 was included for a comparison of the DAD1 protein contents of BHK21 and tsBN7 cells.

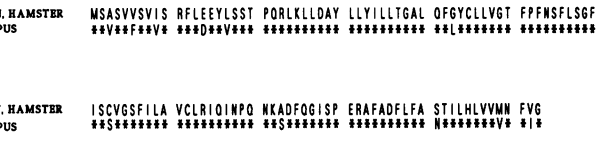

FIG. 9. Comparison of the primary structures of human, hamster, and *Xenopus* DAD1 proteins. The asterisks indicate identical amino acids.

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REFERENCES

1. **Borzillo, G. V., K. Endo, and Y. Tsujimoto.** 1992. Bcl-2 confers growth and survival advantage to interleukin 7-dependent early pre-B cells which become factor independent by a multistep process in culture. Oncogene **7**:869–876.
2. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7**:2745–2752.
3. **Ellis, R. E., J. Yuan, and R. Horvitz.** 1991. Mechanisms and functions of cell death. Annu. Rev. Cell Biol. **7**:663–698.
4. **Ghibelli, L., C. Nosseri, S. Oliverio, M. Piacentin, and F. Antnori.** 1992. Cycloheximide can rescue heat-shocked L cells from death by blocking stress-induced apoptosis. Exp. Cell Res. **201**:436–443.
5. **Hayakawa, H., G. Koike, and M. Sekiguchi.** 1990. Expression and cloning of complementary DNA for a human enzyme that repairs O^6-methylguanine in DNA. J. Mol. Biol. **213**:739–747.
6. **Ishida, Y., Y. Agata, K. Shibahara, and T. Honjo.** 1992. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO J. **11**:3887–3895.
7. **Kozak, M.** 1984. Compilation and analysis of sequences upstream from the translation start site in eukaryotic mRNA. Nucleic Acids Res. **12**:857–872.
8. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
9. **Nishimoto, T., and C. Basilico.** 1978. Analysis of a method for selecting temperature sensitive mutants of BHK cells. Somatic Cell Genet. **4**:323–340.
10. **Nishimoto, T., E. Eillen, and C. Basilico.** 1978. Premature chromosome condensation in a ts DNA^- mutant of BHK cells. Cell **15**:475–483.
11. **Nishimoto, T., T. Sekiguchi, R. Kai, K. Yamashita, T. Takahashi, and M. Sekiguchi.** 1982. Large-scale selection and analysis of temperature-sensitive mutants for cell reproduction from BHK cells. Somatic Cell Genet. **8**:811–824.
12. **Nishitani, H., M. Ohtsubo, K. Yamashita, H. Iida, J. Pines, H. Yasuda, Y. Shibata, T. Hunter, and T. Nishimoto.** 1991. Loss of RCC1, a nuclear DNA-binding protein, uncouples the completion of DNA replication from the activation of cdc2 protein kinase and mitosis. EMBO J. **10**:1555–1564.
13. **Ohtsubo, M., R. Kai, N. Furuno, T. Sekiguchi, M. Sekiguchi, H. Hayashida, K. Kuma, T. Miyata, S. Fukushige, T. Murotsu, K. Matsubara, and T. Nishimoto.** 1987. Isolation and characterization of the active cDNA of the human cell cycle gene (RCC1) involved in the regulation of onset of chromosome condensation. Genes Dev. **1**:585–593.
14. **Pezzella, F., A. G. D. Tse, J. L. Cordell, K. A. F. Pulford, K. C. Gatter, and D. Y. Mason.** 1990. Expression of the bcl-2 oncogene protein is not specific for the 14; 18 chromosomal translocation. Am. J. Pathol. **137**:225–232.
15. **Raff, M. C.** 1992. Social controls on cell survival and cell death. Nature (London) **356**:397–400.
16. **Rebagllati, M. R., D. L. Weeks, R. P. Harvey, and D. A. Melton.** 1985. Identification and cloning of localized maternal RNAs from Xenopus eggs. Cell **42**:769–777.
17. **Takebe, Y., M. Seiki, J. I. Fujisawa, P. Hoy, K. Yokota, K.-I. Arai, M. Yoshida, and N. Arai.** 1988. SRα promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. Mol. Cell. Biol. **8**:466–472.
18. **Uchida, S., T. Sekiguchi, H. Nishitani, K. Miyauchi, M. Ohtsubo, and T. Nishimoto.** 1990. Premature chromosome condensation is induced by a point mutation in the hamster RCC1 gene. Mol. Cell. Biol. **10**:577–584.
19. **Vaux, D. L.** 1993. Toward an understanding of the molecular mechanisms of physiological cell death. Proc. Natl. Acad. Sci. USA **90**:786–789.
20. **Vaux, D. L., H. L. Aguila, and I. L. Weissman.** 1992. Bcl-2 prevents death of factor-deprived cells but fails to prevent apoptosis in targets of cell mediated killing. Int. Immunol. **4**:821–824.
21. **Vaux, D. L., I. L. Weissman, and S. K. Kim.** 1992. Prevention of programmed cell death in *Caenorhabditis elegans* by human bcl-2. Science **258**:1955–1957.
22. **Walker, N. I., B. V. Harmon, G. C. Gobe, and J. F. R. Kerr.** 1988. Patterns of cell death. Methods Achiev. Exp. Pathol. **13**:18–54.
23. **Watanabe, M., N. Furuno, M. Goebl, M. Go, K. Miyauchi, T. Sekiguchi, C. Basilico, and T. Nishimoto.** 1991. Molecular cloning of the human gene, CCG2, that complements the BHK-derived temperature-sensitive cell cycle mutant tsBN63: identity of CCG2 with the human X chromosomal SCAR/RPS4X gene. J. Cell Sci. **100**:35–43.
24. **Woods, A., T. S. Sherwin, R. Sasse, T. H. MacRae, A. J. Bains, and K. Gull.** 1989. Definition of individual components within the cytoskeleton of Trypanosoma brucei by a library of monoclonal antibodies. J. Cell Sci. **93**:491–500.
25. **Wyllie, A. H., J. F. R. Kerr, and A. R. Currie.** 1980. Cell death: the significance of apoptosis. Int. Rev. Cytol. **68**:251–306.