学位論文

Molecular properties and function of DNA-dependent protein kinase in radiation response.

DNA依存性プロテインキナーゼの性質と放射線応答における機能

平成１０年３月博士（理学）申請

東京大学大学院理学系研究科
生物化学専攻

松本　義久

①

MOLECULAR PROPERTIES AND FUNCTION OF DNA-DEPENDENT PROTEIN KINASE IN RADIATION RESPONSE.

DNA依存性プロテインキナーゼの性質と放射線応答における機能

HYPERTHERMIC LABILITY OF DNA-PK AND HYPERTHERMIC RADIOSENSITIZATION.

DNA-PKの温熱不安定性と温熱放射線増感効果の関係についての研究

BY
YOSHIHISA MATSUMOTO

松本　義久

Submitted in partial fulfillment
of the requirement
for the degree
of Ph.D.

Supervised by Prof. Norio Suzuki
Department of Radiation Oncology
Faculty of Medicine
University of Tokyo
Bunkyo-Ku
Tokyo
Japan

Abstract in Japanese

論文の内容の要旨

論文題目　Molecular properties and function of DNA-dependent protein kinase in radiation response.
(DNA依存性プロテインキナーゼの性質と放射線応答における機能)

氏　名　　松　本　義　久

【序】

電離・非電離放射線は、細胞の遺伝情報を担うDNAに種々の傷害を与え、細胞死、変異、ガン化などを引き起こす。X線に代表される電離放射線の場合、DNAの２重鎖切断がこれらの放射線生物学的現象の担い手であると考えられ、細胞は様々な方法でDNA２重鎖切断を修復する。また、細胞は放射線に応答して、G1/S期やG2/M期における細胞周期進行の停止やアポトーシスによる細胞死などを起こす。

DNA依存性プロテインキナーゼ(DNA-PK)は、触媒サブユニットDNA-PKcsとKu抗原ヘテロダイマーKu86およびKu70の３つのポリペプチドから構成されるプロテインキナーゼである。DNA-PKの活性化には2本鎖DNAへの結合が必要であり、また、DNA-PKはp53、Replication protein A(RPA)をはじめとする数多くの核タンパク質をリン酸化する。近年、DNA-PKの３つのサブユニットのうち、DNA-PKcsがV(D)J組換えに欠陥があり、放射線に高感受性を示すマウス*scid* (severe combined immunodeficiency)変異の原因遺伝子であることが報告された。また、Ku86がXRCC5 (X-ray repair cross-complementing)遺伝子産物であり、いくつかの電離放射線感受性変異細胞で欠損していることが報告された。これらのことから、DNA-PKはDNA２重鎖切断の認識や再結合において、重要な役割を担う酵素である可能性が示唆される。

【1.MOLT-4細胞DNA-PKの精製およびサブユニットの分離】

本研究では、まず、ヒト白血病由来MOLT-4細胞核抽出液から、DEAE陰イオン交換カラム2回とDNA-celluloseカラムの計3段階のカラムクロマトグラフィーにより、DNA-PKを精製した。精製したDNA-PK画分は、これまでHeLaおよびRaji細胞から精製が報告されたものと同様に、470kDa、86kDa、70kDaの3本のメインバンドを含んでいた(図1)。470kDa、86kDa、70kDaのバンドはそれぞれ、DNA-PKcs、Ku86、Ku70に対する抗体に反応した。更に、精製DNA-PKをグリセロール濃度勾配中で超遠心することにより、DNA-PKcsとKuサブユニットに分離した。超遠心後の各画分についてリン酸化活性を検討したところ、リン酸化活性はDNA-PKcs画分に顕著に見られた(図2)。リン酸化活性はKu画分のみではほとんど見られなかったが、DNA-PKcsにKuを加えることによりリン酸化活性が促進された(図3)。このことから、DNA-PKcsがリン酸化触媒活性を持ち、Kuサブユニットはこれを増強する役割があることが判明した。

【2.精製DNA-PKおよびサブユニットの温熱処理に対する感受性】

精製したDNA-PKホロ酵素と、分離したサブユニットに対する44℃温熱処理の影響を調べた。まず、DNA-PKホロ酵素を37℃または44℃で処理した後の活性を測定した。44℃では5分間処理から活性の低下が見られたが、37℃では30分間処理してもほとんど活性が低下しなかった(図4)。次に、分離したサブユニットの44℃温熱処理感受性を調べるため、DNA-PKcs画分とKu画分を別々に37℃あるいは44℃で処理した後、両者を混ぜ合わせてリン酸化活性を測定した(図5)。44℃処理したKuと37℃処理したDNA-PKcsを混ぜ合わせたとき活性は、37℃処理したKuと37℃処理したDNA-PKcsを混ぜ合わせたときより顕著に低下していた。一方、37℃処理したKuと44℃処理したDNA-PKcsを混ぜ合わせたときの活性は37℃処理したKuと37℃処理したDNA-PKcsを混ぜ合わせたときとほとんど変わらなかった。更に、44℃処理したKuと44℃処理したDNA-PKcsを混ぜ合わせたときの活性は、44℃処理したKuと37℃処理したDNA-PKcsを混ぜ合わせたときの活性とほぼ同じであった。以上から、DNA-PKは44℃温熱処理に対して不安定であり、Kuサブユニットが温熱不安定性の原因であることが示唆された。

【3.細胞内のDNA-PKの温熱処理に対する感受性】

細胞内のDNA-PKの温熱安定性を調べるために、44℃でさまざまな時間処理した細胞から粗抽出液を調製し、DNA-PK活性を測定した。調べた8種類の細胞のうち、3種類のげっ歯類細胞(V79、FM3A、FSA)では、60分以内の温熱処理で、DNA-PK活性が3分の1あるいはそれ以下に低下した。一方、5種類のヒト細胞(MOLT-4、U937、HL60、MKN-45、A7)は、60分処理までの温熱処理後も未処理のものとほとんど同等のDNA-PK活性を示し、180分処理後においても40%以上の活性を維持していた。これらの結果から、DNA-PKはげっ歯類細胞内で温熱処理に対して不安定であるが、ヒト細胞内では安定であることが示唆された。

【考察】

温熱処理はがん治療に、しばしば放射線と組み合わせて用いられる。温熱処理と放射線を組み合わせる生物学的意義としては、放射線感受性が低いS期細胞が温熱処理に高感受性であること、そして、放射線によって生じた細胞傷害の修復能力が温熱処理によって低下することが挙げられる。DNA切断は放射線によって生じる細胞傷害の中で最も致命的なものと考えられることから、温熱処理による放射線増感作用はDNA切断の修復酵素の不活性化によるところが大きいと考えられている。一方、最近になっていくつかの放射線感受性細胞でDNA-PKのサブユニットが欠損していることが示され、DNA-PKが放射線で生じたDNA傷害、とくに2重鎖切断の認識や修復に重要な役割を担う酵素であることが強く示唆されている。また、一般的にヒト細胞の温熱による放射線増感効果がげっ歯類細胞に比べて小さいという報告がこれまでにいくつかあるが、本研究で、DNA-PKはげっ歯類細胞内で温熱処理に対して不安定であるが、ヒト細胞内では安定であることを示した。これらのことを考え合わせ、本研究で示したDNA-PK、特にそのKuサブユニットの温熱不安定性は、放射線によって生じた2重鎖切断の認識や修復の傷害を介して、温熱処理による放射線増感作用の原因の1つとなっている可能性が示唆された。

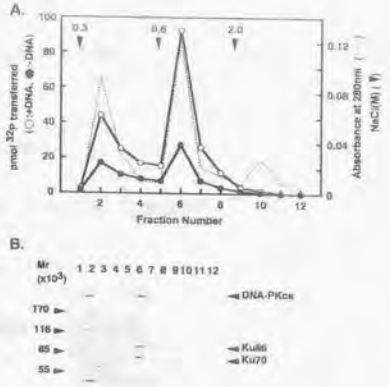


図1.MOLT-4細胞のDNA-PKのDNA-celluloseカラムクロマトグラフィーにおける挙動。A.溶出画分のα-カゼインリン酸化活性。B.SDS-PAGEパターン。

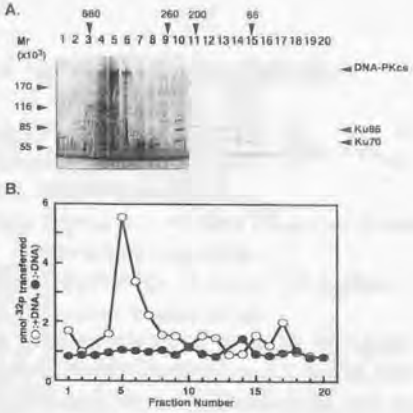
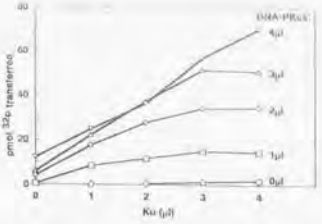


図2.グリセロール濃度勾配中での遠心によるDNA-PKcsとKuサブユニットの分離。A.SDS-PAGEパターン。B.合成ペプチドリン酸化活性。



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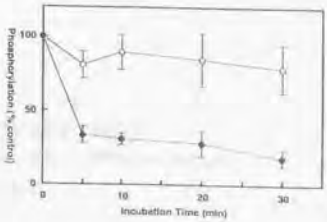

図4.精製DNA-PKホロ酵素を37℃(○)、44℃(●)で各時間前処理した後のリン酸化活性。

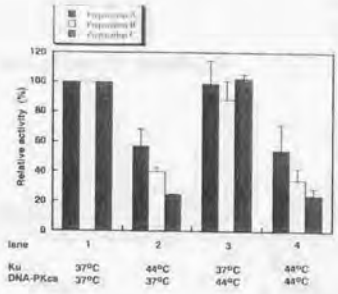

図5.DNA-PKcsとKuを別々に37℃、44℃で前処理後再混合した場合のリン酸化活性。

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Abbreviations

AT: ataxia-telangiectasia.
ATM: ataxia-telangiectasia mutated.
DNA-PK: DNA-dependent protein kinase.
DNA-PKcs: DNA-dependent protein kinase catalytic subunit.
DSB: double-strand break.
DSBR: double-strand break repair.
DTT: dithiothreitol.
PAGE: polyacrylamide gel electrophoresis.
PLDR: potentially lethal damage repair.
RPA: replication protein A.
SDS: sodium dodecylsulfate.
SLDR: sublethal damage repair.
SSB: single-strand break.
TER: thermal enhancement ratio.
XRCC: X-ray repair cross-complementing.

I. INTRODUCTION.

1.1. DNA double-strand break and DNA-dependent protein kinase.

Ionizing and non-ionizing radiation cause various damages on DNA molecules, such as base alteration and single- and double-strand breaks, and biological effects, such as cell killing, mutagenesis and transformation. In the case of ionizing radiation, the double-strand break (DSB) of DNA is considered the main mediator of these radiobiological effects and cells repair DSBs by various mechanism. In response to radiation, cells also arrest at G1/S and/or G2/M boundary or die via apoptosis (1).

DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine protein kinase composed of three subunits of 470kDa, 86kDa and 70kDa, respectively (2-6). 470kDa subunit is the catalytic subunit (DNA-PKcs) (7) and 86kDa and 70kDa subunits (Ku86 and Ku70, respectively) form a heterodimer termed Ku antigen (8,9). DNA-PK binds to and requires ends of double-stranded DNA to be activated (6, 10) and phosphorylates a number of nuclear proteins including p53 (2, 3, 11, 12) and replication protein A (RPA) (13, 14), suggesting its role in rejoining double-strand breaks of DNA and in triggering cellular response to radiation such cell cycle arrest and cell death by apoptosis.

1.2. Possible roles of DNA-PK in double-strand break repair.

DNA-PKcs is defective in the *scid* (severe combined immunodeficiency) mouse (15-17), lacking mature B and T cells due to a defect in V(D)J recombination (18, 19). The *scid* mouse is also hypersensitive to ionizing radiation due to a defect in repair of DSB (20-22). A human glioma cell line MO59J, lacking DNA-PKcs,

also showed radiosensitivity with defective double-strand break repair (23). Ku86 is XRCC5 (X-ray cross-complementing 5) gene product that is defective in X-ray sensitive rodent cell lines including*xrs*-5, -6, and XR-V15B (24, 25). These cell lines are also defective in artificial V(D)J recombination induced by ectopic expression of recombination activating genes RAG1 and RAG2 (24, 25, 26). Additionally, mice deficient for Ku86 or Ku70 gene, established using gene-targeting mutagenesis, is hypersensitive to radiation and defective in V(D)J recobmination (27-30). Mammalian mutants deficient for DNA-PK subunits are listed in Table.1-1., most of which exhibit elevated radiation sensitivity and defective V(D)J recombination. These facts indicate that DNA-PK plays an essential role in repair of radiation induced DSBs and V(D)J recombination.

DNA-PK is supposed to bind to the end of double-strand DNA breaks, to phosphorylate repair enzymes, such as DNA ligases, controlling their activity and localization and to protect DNA ends from nuclease digestion (39). In addition to DNA-PK, XRCC4 and DNA ligase IV are likely to participate in mammalian DSB repair. XR-1 cells, lacking XRCC4 gene, is phenotypically similar to *scid* and *xrs*, being hypersensitive to ionizing radiation with defects in rejoining of DSB and incapable of carrying out V(D)J recombination (26, 40). XRCC4 protein is phosphorylated by DNA-PK *in vitro* and possibly also *in vivo* (41). Additionally, XRCC4 forms complex with and stimulates DNA ligase IV (41, 42). Regarding these facts, XRCC4/DNA ligase IV complex might be a DSB rejoining enzyme cooperating with DNA-PK.

Table 1. Mammalian mutants deficient for DNA-PK.

I) Mutants deficient for DNA-PKcs

Mutant	Species/Origin	Molecular defects	References
Scid mouse	Mouse	Truncated protein lacking C-terminal 83aa	15-17, 31-33
V3	Chinese hamster/CHO-AA8	Decreased transcript	16
MO59J	Human/Glioma biopsy	Decreased transcript	23
Equine *scid*	Horse	Decreased transcript	34
IRS20	Chinese hamster/CHO-10B2	Unknown	35
SX-9	Mouse/FM3A	Unknown	35

II) Mutants deficient for Ku86

Mutant	Species/Origin	Molecular defects	References
xrs-1	Chinese hamster/CHO-K1	Decreased transcript	24, 36
xrs-4	Chinese hamster/CHO-K1	Frameshifted and truncated protein of 287 amino acids	24, 36
xrs-5	Chinese hamster/CHO-K1	Decreased transcript	24, 36
xrs-6	Chinese hamster/CHO-K1	Frameshifted and truncated protein of 25 amino acids	24, 36
xrs-7	Chinese hamster/CHO-K1	Decreased transcript	24, 36
XR-V15B	Chinese hamster/V79	Protein with in-frame deletion of codon 372-417	25, 37
XR-V9B	Chinese hamster/V79	Protein with in-frame deletion of codon 267-350	25, 37
sxi-3	Chinese hamster/V79	Decreased transcript	38
Knock-out mouse	Mouse	Homologous recombination-mediated gene disruption	27, 28

III) Mutants deficient for Ku70

Mutant	Species/Origin	Molecular defects	References
Knock-out mouse	Mouse	Homologous recombination-mediated gene disruption	29, 30

1.3. Possible roles of DNA-PK in signal transduction of DNA damage.

DNA-PK recognizes serine or threonine residues either preceded or followed by glutamine (12, 43, 44) and is known to phosphorylate a number of proteins that participate in transcription, cell cycle control and/or cell death (Table.1-2). Among these proteins, p53, RPA and c-Abl responses to ionizing radiation and are thought to transduce DNA damage signals at downstream of DNA-PK.

p53: p53 is a product of tumor suppressor gene and implicated in cell cycle arrest and in apoptosis. These functions of p53 are most likely mediated through transactivation of target genes such as cyclin-dependent kinases inhibitor $p21^{WAF1/Cip1/Sdi1}$ and apoptosis promotor Bax (57). p53 protein accumulates through posttranscriptional mechanism(s) following irradiation (58) with subsequent increase in transcript of p21 (59). Serines 15 and 37 of human p53 and serine 18 of murine p53 *in vitro* by DNA-PK (11, 12) and in cell following irradiation (60). Phosphorylation of p53 by DNA-PK interfere with binding to Mdm2, which inhibits transcirptional activation by p53 (61, 62) and promotes degradation by proteasome of p53 (63, 64). Therefore, in response to DSB induced by ionizing radiation, DNA-PK might upregulate and stabilize p53 through phosphorylation, leading to expression of genes related to cell cycle arrest and apoptosis. Additionally, since the DNA-PK phosphorylation sites on p53 are located within the domain through which p53 interacts with replication and repair protein RPA and with general transcription factor TAFs (TBP associated factors), DNA-PK might also modify interaction between p53 and these proteins (57).

Table 2. Proteins phosphorylated by DNA-PK.

Protein	Function	Reference
p53	Transcription factor, controling cell cycle and/or cell death	2, 3, 11, 12, 62
Replication protein A (RPA)	DNA replication and nucleotide excision repair	13, 14, 45, 46
c-Abl	Protein tyrosine kinase, cell proliferation control	47, 48
XRCC4	DNA double-strand break repair and V(D)J recombination, interacting with DNA ligase IV.	41
SV40 T antigen	Viral DNA replication and transcription	48
RNA polymerase II largest subunit	Transcription	49
Sp1	Transcription factor	50
c-Jun	Transcription factor	44
c-Myc	Transcription factor	51
Serum response factor (SRF)	Transcription factor	52
TATA-box binding protein (TBP)	Basal transcription factor	53, 54
TFIIB	Basal transcription factor	54
Glucocorticoid receptor	Transcription factor	55, 56
Hsp90	Stress response	43

Replication protein A (RPA; also termed HSSB): RPA, a heterotrimeric protein composed of 70kDa, 34kDa and 14kDa subunits, plays essential roles in DNA replication and in nucleotide excision repair. These functions of RPA is thought to be mediated through interaction with single-stranded region of DNA and with other proteins including DNA polymerases, XPA and ERCC1. Since RPA interact also with Rad51, a homologous recombination factor, and with p53, it may have additional roles in recombination and/or in transcription. DNA-PK phosphorylates *in vitro* the 34kDa subunit of RPA (13, 14, 45, 46), which is phosphorylated in S-phase (65) and following irradiation *in vivo* (66, 67). Though the significance of this phosphorylation is not well clarified, DNA-PK might regulate function or localization of RPA in DNA replication, transcription or repair of DSB through phosphorylation.

c-Abl: c-Abl tyrosine kinase interacts with DNA-PKcs constitutively and with Ku following γ-irradiation and the interaction between c-Abl and DNA-PK is enhanced by γ-irradiation (47). c-Abl-DNA-PK interaction is thought to be mediated through SH3 (Src homology domain 3) of c-Abl and a proline-rich region located within kinase homology domain of DNA-PK (68). DNA-PK phosphorylates and stimulates kinase activity of c-Abl *in vitro*. The kinase activity of c-Abl increases following γ-irradiation and the increment is smaller in *scid* cells, lacking DNA-PKcs, than wild-type. Since c-Abl is implicated in ionizing radiation-induced cell cycle arrest and apoptosis (69, 70), DNA-PK might trigger these response through upregulation of c-Abl. Additonally, since c-Abl phosphorylates DNA-PKcs following γ-irradiation and induce its dissociation from DNA, c-Abl might negatively regulate DNA-PK as a feedback mechanism.

1.4. Homologues and relatives of DNA-PK subunits.

Recent studies indicate that eukaryotic cells repair DSBs either via homologous recombination or via non-homologous end joining and that these mechanisms for DSB repair might be conserved throughout eukaryote (71). Thus studies on DNA-PK related molecules in yeast or other organisms are indicative of DNA-PK function in mammal.

Homologues of Ku proteins: Homologues of Ku proteins are identified in mammal (human, hamster, mouse), fry (*Drosophila melanogaster*) and yeast (*Saccharomyces cerevisiae*). Ku70 and Ku86 homologue in yeast is well-studied and termed HDF1 and HDF2, respectively, for high-affinity DNA-binding factor (72-75). *Hdf1* and *hdf2* mutant show elevated radiosensitivity in *rad52* background (75-77) and defects in illegitimate recombination (78) and in recircularization of restriction enzyme-digested plasmids (75, 77). Thus HDF1/2 are thought to participate in non-homologous end-joining, while RAD52 participate in homologous recombination. Mutant of DNL4 gene, the yeast homologue of DNA ligase IV show similar phenotype to hdf1 or hdf2, indicating cooperation between Ku and DNA ligase IV in DNA recombination and/or repair is conserved throughout eukaryote (79, 80). Additionally, Hdf1p was found to interact with a transcriptional silencing factor Sir4p in two-hybrid analysis and *sir2*, *sir3* or *sir4* mutant were silmilar to *hdf* mutants regarding defective illegitimate recombination and recircularization of linearized plasmids and elevated radiosensitivity in *rad52* background (81). It would be interesting whether Ku and/or DNA-PKcs in mammal also cooperate with silencer proteins.

Drosophila Ku70 homologue is known as IRBP (inverted-repeat binding protein) that binds to inverted repeats in transposable P elements and *mus309* mutant, lacking IRBP, show large deletion after P-element excision and elevated sensitivity to methylmethane-sulfonate (82,83). These facts indicate that functions of Ku proteins in DNA repair and site-specific recombination, such as V(D)J recombination and P-element transposition, are conserved throughout eukaryote.

Relatives of DNA-PKcs: Homologues of DNA-PKcs are described only in mammal. However, DNA-PK-like activity was also detected in extracts from *Xenopus* egg and *Drosophila* embryo (84). Thus, DNA-PKcs might be conserved throughout animal kingdom or throughout eukaryote. The putative kinase domain at the C-terminus of DNA-PKcs is related to phosphatidylinositol (PI) kinases and, more closely to, ATM, which is considered reponsible for human genetic disorder ataxia-telangiectasia (7, 85). Ataxia-telangiectasia patients display symptoms including immunodeficiency, chromosomal instability and predisposition to cancer, indicating possible roles of ATM in recombination and genome surveillance (86). Additionally, cultured cell lines from AT patient show hypersensitity to ionizing radiation with impaired cell cycle arrest and p53 induction (87-89), indicating that ATM might also be required for signal transduction of radiation-caused DNA damage (86). The role of ATM in radiation response might be similar to or partially overlapping with that of DNA-PKcs, as indicated by the fact that radiation induced phosphorylation of RPA is partially impaired in AT cells (66) and the evidence that ATM binds to, phosphorylates and stimulates c-Abl (90, 91).

Proteins related to DNA-PKcs and ATM, collectively termed ATM family proteins, include Mec1p/Esr1p (92, 93) and Tel1p (94, 95) from *Saccharomyces cerevisiae*, Rad3p (96, 97) from *Schizosaccharomyces pombe* and Mei41p (98) from *Drosophila melanogaster*, all of which are implicated in recombination and/or cell cycle checkpoint. Additionally, there is another DNA-PKcs- and ATM-related molecule, termed ATR, in human, which might have function in meiotic recombination (90, 99, 100).

1.5. The aim of this study.

Since DNA-PK is considered a critical enzyme in repair and/or signal transduction of DSB, the inhibition or inactivation of this enzyme may result in cellular radiosensitization. Hyperthermia has cell killing effect either by itself or synergistically with radiation, and thus is used to treat cancer mostly combined with radiation (1). One of the biological basis for the combined use of hyperthermia and radiation is complementary patterns of sensitivity through the cell cycle: the radioresistant S phase cells are sensitive to hyperthermia (1). Additionally, radioresistant hypoxic cells are no more resistant or even more sensitive to radiation than aerated cells (1). Furthermore, hyperthermia has radiosensitizing effect possibly due to inhibition of repair of radiation induced damage, as there are lines of evidence indicating that hyperthermia inhibits rejoining of single- or double-strand breaks (1, 101, 102, 103).

On the basis of these facts, I studied hyperthermic stability of DNA-PK, in order to examine the possibility that hyperthermic radiosensitization be mediated through effect on this enzyme.

II. MATERIALS AND METHODS.

2.1. Reagents.

Reagents were obtained from Wako Pure Chemical or Nakarai Tesque, unless otherwise indicated, except for the following: RPMI1640 medium from Life Technologies (Grand Island, New York, USA), calf bovine serum from Hyclone (Logan, Utah, USA), penicillin potassium from Banyu (Tokyo, Japan), streptomycin sulfate from Meiji (Tokyo, Japan), DEAE Bio-Gel A from Bio-Rad (Richmond, California, USA), DNA-cellulose from Pharmacia (Uppsala, Sweden), α-casein from United states Biochemicals (Cleaveland, Ohio, USA), and [γ-^{32}P]ATP from NEN (Boston, Massachusetts, USA). Synthetic peptide, used as a substrate for DNA-PK, is produced by myself or Iwaki Glass (Funabashi, Japan) by standard Fmoc method.

2.2. Cell culture

Cell lines used for this study were grown at 37^{0}C in humidified atmosphere with 5% CO_2. V79 chinese hamster lung fibroblast, FM3A Cio^{r}-3 murine mammary carcinoma and A7 human glioma cells were cultured in Minimal Essential alpha Medium (MEM) supplemented with 10% calf bovine serum. MOLT-4 human T cell leukemia and MKN-45 human stomach cancer cells were cultured in RPMI-1640 medium supplemented with 10% calf bovine serum. U937 monocytic lymphoma and HL60 promyelotic leukemia cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum. FSA murine fibrosarcoma cells were cultured in McCoy's 5A medium supplemented with 10% CBS. All media were supplemented with 10^{5}U/l of penicillin potassium and 100mg/l of streptomycin.

Cells were harvested by tripsinization, if necessary, and centrifugation (350xg for 5 min) followed by washing twice with ice-cold Tris-buffered saline (10mM Tris-HCl, pH 7.4, 2mM $MgCl_2$, 140mM NaCl) and were stored at -70°C until use. Part of MOLT-4 cells, used for DNA-PK purification, were kindly provided by Dr. Takeuchi (Fujisaki Institute of Hayashibara Biomedical Laboratories Inc., Okayama, Japan).

2.3. DNA-PK purification.

MOLT-4 cell nuclei were prepared from 2-10x10^9 cells as described (104). Nuclei were resuspended in buffer A (20mM HEPES-NaOH, pH 7.9, 400mM KCl, 1mM EDTA, 1mM EGTA, 0.02% Tween 20, 10% glycerol, 1mM dithiothreitol (DTT), 1mM PMSF (phenylmethylsulfonyl fluoride), 1μg/ml of leupeptin, pepstatin and antipain, respectively) and agitated with a stirring bar for 30 min followed by centrifugation at 100,000g for 60 min. The supernatant nuclear extract (30ml) was passed through the first DEAE Bio-Gel A column and dialyzed against buffer B (20mM Tris-HCl, pH 7.5, 1mM EDTA, 10% glycerol, 50mM NaCl, 1mM DTT, 1mM PMSF, 1μg/ml of leupeptin, pepstatin and antipain, respectively). Dialysate was applied to the second DEAE Bio-Gel A and eluted with buffer B with increasing NaCl concentration linearly from 0.05M to 0.3M. DNA-PK was eluted with 0.14-0.17M NaCl. The DNA-PK fractions were passed through NAP-25 column equilibrated with buffer C (20mM HEPES-NaOH, pH 7.2, 1mM $MgCl_2$, 15% glycerol, 200mM NaCl, 1mM DTT, 1mM PMSF, 1μg/ml of leupeptin, pepstatin and antipain, respectively) and finally loaded onto native DNA-cellulose column (1ml). Absorbed protein was eluted into 12 fractions (1ml each) by stepwise increase of NaCl concentration in

buffer C(Fig.2). Fraction 6, 0.6M NaCl eluate was used as the purified DNA-PK holoenzyme for further studies.

2.4. Separation of DNA-PKcs and Ku subunits by glycerol gradient ultracentrifugation.

Two hundred µl aliquot of purified DNA-PK (fraction 6 of native DNA-cellulose column chromatography) was mixed with 100µl of water, layered on 5ml of 15-35% glycerol gradient in buffer D (20mM HEPES-NaOH, pH 7.2, 1mM $MgCl_2$, 100mM NaCl, 1mM DTT) and centrifuged at 40,000rpm (160,000g) for 21h using an SW50.2 roter (Beckman, Palo Alto, California, USA). Twenty fractions of 250µl each were collected, and DNA-PKcs and Ku subunits were found separated in fractions 5 and 6 and fractions 11 through 17, respectively (Fig.5).

2.5. Preparation of crude cell extract.

Pellets of 3 to $10x10^6$ cells were washed twice with ice-cold Tris-buffered saline (10mM Tris-HCl (pH 7.4), 2mM MgCl2, 140mM NaCl) and were resuspended in 30 to 100µl of extraction buffer (20mM HEPES-NaOH (pH 7.9), 400mM KCl, 1mM EDTA, 1mM EGTA, 10% glycerol, 0.02% Tween 20, 1mM PMSF, 1µg/ml of leupeptin, pepstatin and antipain, respectively) followed by three-rounds of freezing in liquid nitrogen bath and thawing in water bath at 30°C. The crude cell extract obtained after incubation for 30 min at 4°C and centrifugation at 15,000g for 7 min to remove cell debris. The protein concenration of crude cell extract was determined using BCA protein assay kit and bovine serum albumin as a standard (Pierce, Rockford, Illinois, USA) and diluted to 1.0 or 3.3mg/ml.

2.6. Protein kinase assay.

DNA-PK activity was assayed using a synthetic peptide p53-#15 (EPPLSQEAFADLWKK) (23) as a substrate except indicated otherwise.

Filter binding protocol: In section 3.1 and 3.2., the reaction mixture (total volume 20μl) contained 20mM HEPES-NaOH, pH 7.2, 100mM NaCl, 5mM $MgCl_2$, 50μM [γ-^{32}P]ATP, 5μg peptide substrate, 1mM DTT and 0.5mM each of NaF and β-sodium glycerophosphate and 0.5 to 5 μl of enzyme fraction was added. The reaction mixture was incubated at 37°C for 10 min and the reaction was stopped by the addition of equal volume of 30% acetic acid. The reaction mixture was spotted onto a P81 phosphocellulose paper disk (Whatman, Maidstone, England), washed in 15% acetic acid and counted in a liquid scintillation counter (Beckman). The amount of phosphate incorporated to peptide was calculated by comparing the radioactivity on paper disk to that of known amount, i.e. 10pmol, of ATP.

In the Fig.1 and 2 experiment, 50μg α-casein in a total volume 50μl was used as a substrate. After incubation at 37°C for 10 min, a 40μl aliquot was spotted onto a square 3MM paper (Whatman), washed with 5% trichloroacetic acid solution supplemented with 0.5% sodium pyrophosphate and counted in a liquid scintillation counter.

Electrophoretic resolution protocol: In section 3.3., I devised a new protocol, modifying "pull-down assay protocol" described by Finnie et al. (84). Fifteen μl aliquot of crude cell extract, containing 15 to 50μg, was mixed with 6μg wet weight of native DNA-cellulose (Pharmacia, Uppsala, Sweden) suspended in 30μl of HK buffer (20mM HEPES-NaOH (pH 7.2), 50mM KCl, 1mM

$MgCl_2$, 15% glycerol, 1mM DTT), incubated for 30 min at 4°C and centrifuged at 15,000g for 2 min. The precipitate was washed twice with 1ml of HK buffer, resuspended in 20μl of kinase buffer (20mM HEPES-NaOH (pH 7.2), 150mM KCl, 5mM $MgCl_2$, 50μM [γ-^{32}P]ATP (7.4kBq/nmol), 0.25mg/ml synthetic peptide p53-#15, 1mM DTT, 0.5mM sodium β-glycerophosphate, 0.5mM NaF) and incubated at 37°C for10min. The reaction mixture was fractionated through 15% polyacrylamide gel following standard SDS-PAGE protocol, instead of spotting onto phosphocellulose paper in the protocol of Finnie et al. (84). The spots of phosphorylated peptide were analyzed by scanning dried gel using phosphor imager BAS2000 (Fuji) and the amount of phosphate incorporated to peptide was calculated by comparing the radioactivity of phosphorylated peptide spots to that of known amount, i.e. 10pmol, of ATP. Part of these experiments, i.e. those on FM3A Clor-3, FSA, MKN-45 and A7 were carried out by Ms. N. Umeda.

2.7. Antibodies and Western blotting.

Rabit antisera AHP316, AHP317 and AHP318, reactive to Ku70, Ku86 and DNA-PKcs respectively, were purchased from Serotec (Oxford, UK). Ku auto-antiserum, from a scleroderma-polymyositis overlap syndrome patient, which was immunoreactive to both Ku86 and Ku70 antigen (8, 9), was a gift from Dr. Mimori (Keio Universtiy, Tokyo, Japan). Mouse monoclonal antibody, specifically reactive to Ku86 subunit, was a gift from Dr. Okumura (Shionogi Institute for Medical Science, Osaka, Japan).

For western blotting, protein spots after SDS-PAGE were electrophoretically transferred using semidry transfer system (ATTO, Tokyo, Japan) onto a polyvinylidene difluoride (PVDF)

membrane (Immobilon, Millipore, Bedford, Massachusetts, USA). The membrane was probed with antiserum or antibody described above and visualized with HRP-1000 (Konica, Tokyo, Japan).

2.8. X-ray irradiation and hyperthermic treatment.

Hyperthermic treatment (107) was carried out by submerging the culture flask or Eppendorf tube in a water bath (Ikemoto Rika, Tokyo, Japan) set at 44°C with the precision within 0.05°C. Cells were irradiated using HF-350 (Shimadzu, Kyoto, Japan) at 1.4Gy/min (operated at 200kV, 20mA with filters of 0.5mm Cu and 1.0mm Al) at room temperature. When combined with hyperthermia, X-ray treatment was given following hyperthermia within 30 min.

2.9. Colony formation assay for cell survival.

Cell survival of V79, FSA, A7 or MKN-45 cells was determined as their colony forming ability on plastic dish. Following hyperthermia and/or irradiation, cells were trypsinized, recovered with medium, counted, diluted and plated onto 60mm dishes in triplicate. After incubation for 6 to 9 days for V79 cells and 12 to 16 days for other cells, cells were stained with crystal violet and colonies were counted. Plating efficiency (P.E.) and surviving fraction (S.F.) were calculated as follows:

P.E. = 100 x the mean number of colony formed divided by the number of cells plated onto dises,

S.F. (%) = P.E. of cells treated with hyperthermia and/or irradiation divided by P.E. of control.

Cell survival of MOLT-4, U937, HL60, FM3A was determined as their colony forming ability in soft agar. Following hyperthermia

and/or irradiation, cell suspension after appropriate dilution was mixed with medium containing 15% serum and 0.16% agarose (Nakarai Tesque, Kyoto, Japan). After incubation for 6 to 8 days for FM3A, 12 to 16 days for MOLT-4 and U937 and 21 to 25 days for HL60 cells, visible colonies were counted and P.E. and S.F. were calculated as indicated above.

X-ray dose-survival curves were fitted to linear-quadratic (LQ) model equation, $S.F. = \exp(-\alpha D - \beta D^2)$, by minimal square method as calculated by software "Kaleidagraph". D_{10}, the X-ray dose giving 10% survival, was calculated from the fitted LQ equation. Thermal enhancement ratio (TER) is calculated as D_{10} of unheated cells divided by that of heated cells.

III. RESULTS

3.1. Purification and characterization of DNA-PK from MOLT-4 nuclei.

3.1.1. Purification of DNA-PK from MOLT-4 nuclei.

Table.3 summerizes purification steps and the results. In the second DEAE Bio-Gel A column, DNA-PK activity was eluted with 0.14 to 0.16M NaCl (Fig. 1, fractions 17 through 24) and in the final native DNA-cellulose column chromatography, DNA-PK activity was eluted with 0.3 to 0.6M NaCl eluate (Fig.2, fractions 2 through 8). Fraction 6, the 0.6M NaCl eluate, from native DNA-cellulose column chromatography (Fig.2B, lane 6) contained three major polypeptide bands of >170kDa, 85kDa and 70kDa. After Western blotting, rabbit antiserum against DNA-PKcs, Ku86 and Ku70 reacted with bands of >170kDa, 85kDa and 70kDa, respectively, (Fig.3) indicating that these were three subunits of DNA-PK, i.e. DNA-PKcs, Ku86 and Ku70. Additionally, Ku auto-antiserum reacted with 85kDa- and 70kDa-bands, however, anti-Ku86 monoclonal antibody reacted only with 85kDa bands, supporting that 85kDa- and 70kDa-bands are the two subunits of Ku antigen (data not shown).

Purified DNA-PK exhibited maximal activity in pH between 7.2 to 9.3 (Fig.4A) and in NaCl concentration between 165 and 215mM (Fig.4B). Glycerol concentration higher than 10% v/v inhibited DNA-PK (Fig.4C). Additionally, single-stranded DNA did not activate but inhibited DNA-PK (Fig.4D). In agreement with the idea that Ku is a component of DNA-PK, antiserum against Ku86 partially inhibited DNA-PK activity (Fig.4E). These characteristics of DNA-PK from MOLT-4 cells were similar to those purified from HeLa and Raji cells (2-6, 51, 105, 106).

3.1.2. Separation of DNA-PKcs and Ku subunits by glycerol gradient ultracentrifugation.

DNA-PK holoenzyme (fraction 6 of Fig.2B) was further separated into DNA-PKcs and Ku subunits by ultracentrifugation in a glycerol gradient. Most of DNA-PKcs was present in fractions 5 and 6, while most of Ku86 and Ku70 were present in fraction 11 through 17 as shown in Fig.5A. Kinase activity was found in DNA-PKcs fractions (Fig.5B), and Ku fraction itself had little or no kinase activity, however, the addition of Ku to DNA-PKcs enhanced kinase activity (Fig.6).

Table 3. Purification of DNA-PK from high salt extract of MOLT-4 cell nuclei.

Purification Step	Protein (mg)	α-casein phosphorylation(pmol/min)			Specific Activity (pmol/min/mg)	Purification (fold)
		+DNA[a]	-DNA[b]	Net[c]		
Nuclear extract	383	30800	26300	4400	11.5	1
DEAE flow through	314	50200	36100	14100	44.9	3.9
DEAE gradient	19.7	36700	13700	23000	1170	100
DNA-cellulose	1.03	5500	995	4500	4368	380

[a] Phosphorylation in the presence of 20ng/ μl sonicated salmon sperm DNA.

[b] Phosphorylation in the absence of DNA.

[c] Phosphorylation in the presence of 20ng/ μl sonicated salmon sperm DNA minus phosphorylation in the absence of DNA.

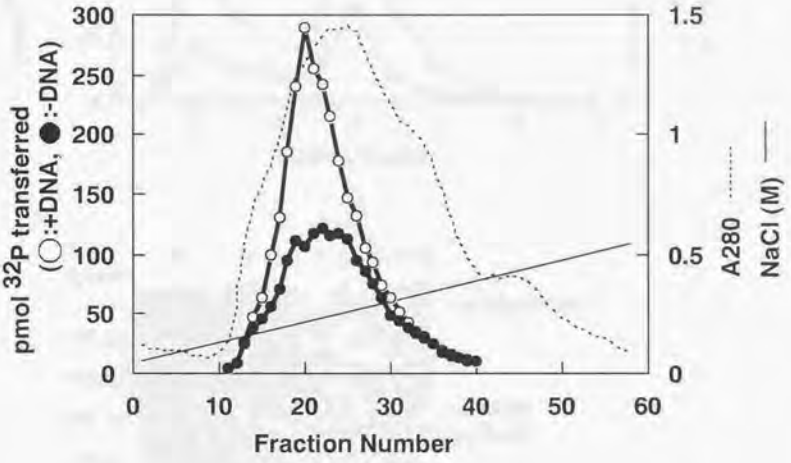

Figure1. The elution profiles of the second DEAE-Bio Gel A column chromatography.
Protein kinase activity of 5μl aliquot from each fraction was determined using α-casein as a substrate in the presence (○) or absence (●) of 20ng/μl of sonicated salmon sperm DNA at 37°C for 10 min. Thin line and dotted line show NaCl concentration and UV absorbance at 280nm, respectively.

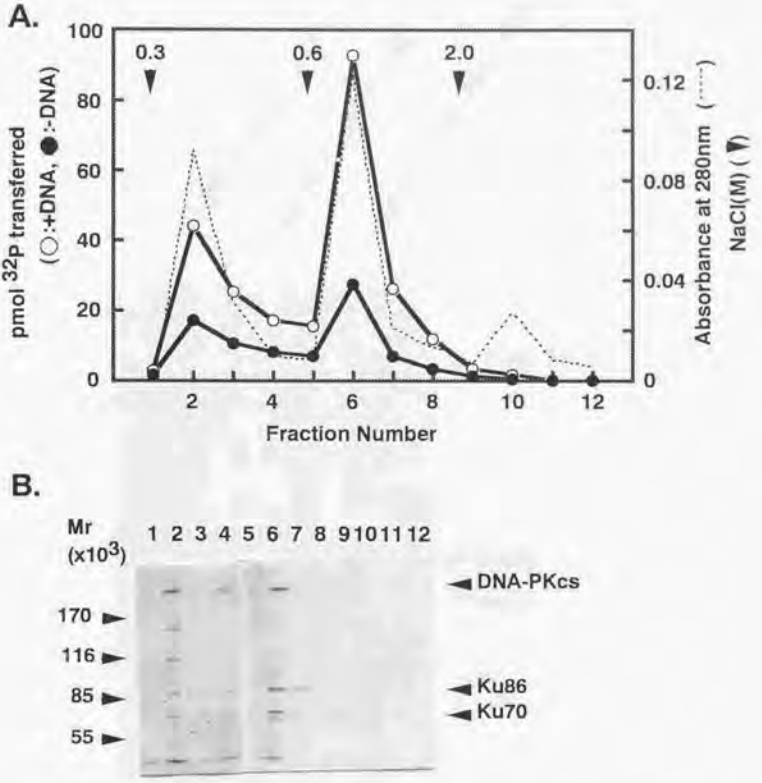

Figure2 . The elution profiles of native DNA-cellulose column chromatography.
A, Protein kinase activity of 5μl aliquot from each fraction was determined using α-casein as a substrate in the presence (○) or absence (●) of 20ng/μl of sonicated salmon sperm DNA at 37°C for 10 min. NaCl concentration is indicated with arrow heads and broken line shows UV absorbance at 280nm. B, SDS-PAGE paterns of 10μl aliquot from each fraction, visualized by Coomassie Brilliant Blue staining. Fraction numbers are indicated at the top. The positions of DNA-PKcs, Ku86 and Ku70 are indicated by arrows.

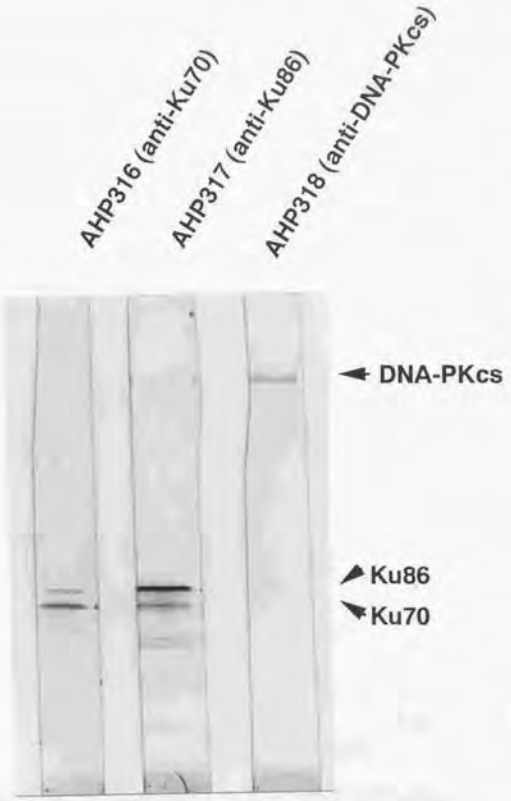

Figure3 . Western blotting analysis of purified DNA-PK. Purified DNA-PK (fraction 6 of Figure2) was separated through SDS-PAGE and transblotted to polyvinylidene difluoride membrane followed by detection using antiserum AHP316, AHP317 or AHP318.

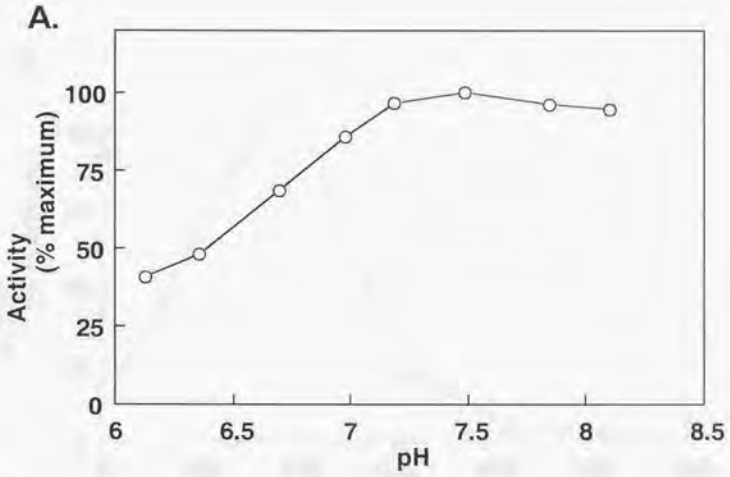

Figure4 . Characteristics of purified DNA-PK.
A, DNA-PK activity in various pH. The kinase activity of purified DNA-PK was assayed using synthetic peptide as a substrate at 37°C for 10 min with 20ng/μl of sonicated salmon sperm DNA in reaction buffer with varying pH. Activity is expressed as the percentage of maximum activity, i.e. that in pH 7.5.

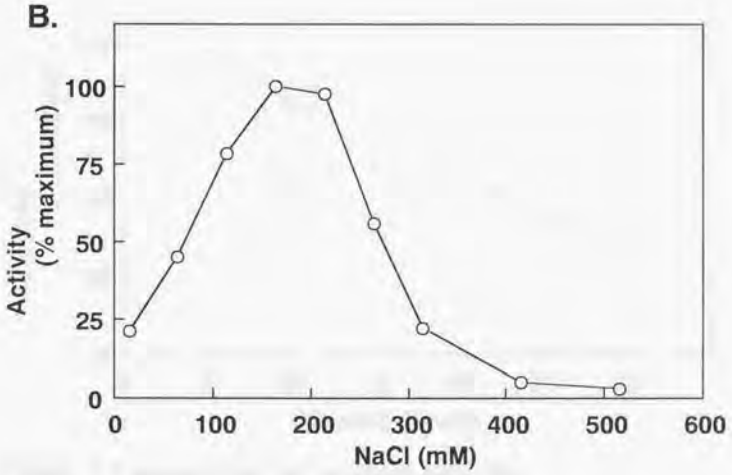

Figure4 . Characteristics of purified DNA-PK.
B, DNA-PK activity in various NaCl concentration. The kinase activity of purified DNA-PK was assayed as in A, except that reaction buffer contained varying concentration of NaCl. Activity is expressed as the percentage of maximum activity, i.e. that in 165mM of NaCl.

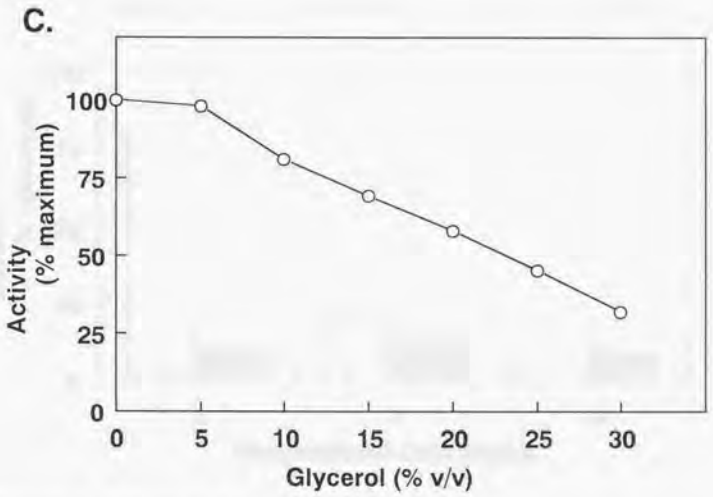

Figure 4. Characteristics of purified DNA-PK.
C, DNA-PK activity in various glycerol concentration. The kinase activity of purified DNA-PK was assayed except that reaction buffer contained varying concentration of glycerol. Activity is expressed as the percentage of that in the absense of glycerol.

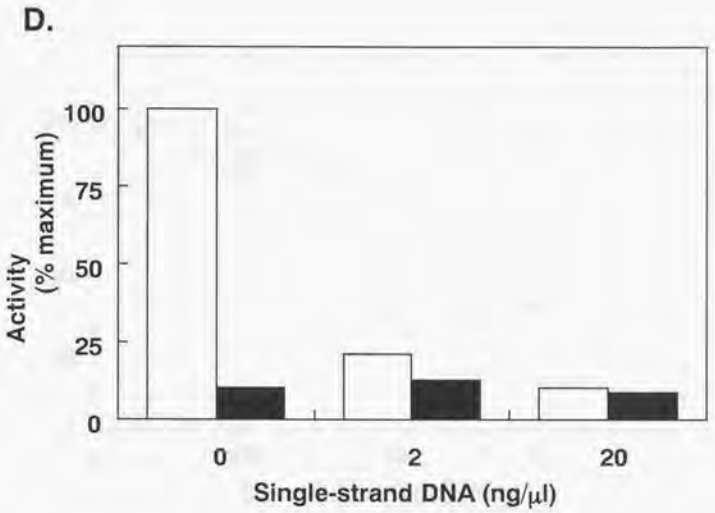

Figure 4. Characteristics of purified DNA-PK.
D, Effect of single-standed DNA on DNA-PK activity. The kinase activity of purified DNA-PK was assayed using synthetic peptide as a substrate at 37°C for 10 min in the presence or absence of double-strand DNA (BamHI-digested pCV108 DNA) or single-strand DNA (M13 phage DNA). Open and closed bar shows phosphorylation in the presence and absence of double-strand DNA, while the concentration of single-strand DNA is indicated at the bottom. Activity is expressed as the percentage of maximum, i.e. that in the presence of 20ng/μl of double-stranded DNA and no single-stranded DNA.

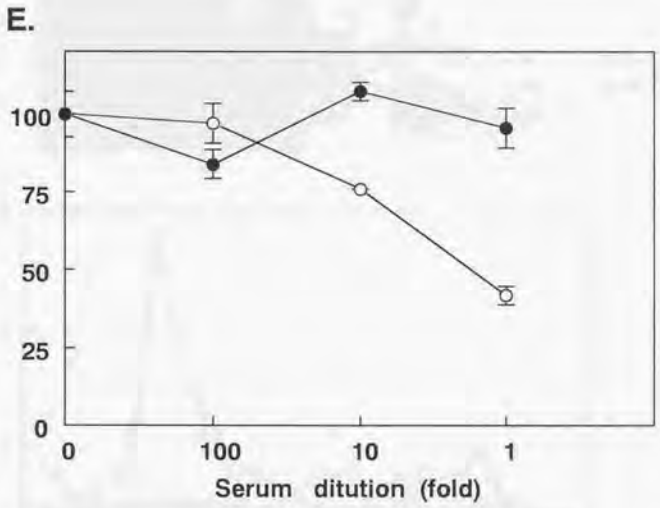

Figure 4. Characteristics of purified DNA-PK.
E, DNA-PK activity after preincubation with anti-Ku86 serum AHP317. Purified DNA-PK was preincubated with 1 μl of diluted AHP317 (○) or unimmunized rabbit serum (●), on ice for 60 min, and assayed for kinase activity using synthetic peptide as a substrate at 37°C for 10 min with 20ng/μl of sonicated salmon sperm DNA. Activity is expressed as the percentage of control, i.e. that after preincubation without serum, with the standard deviations of duplicate assays indicated by error bars.

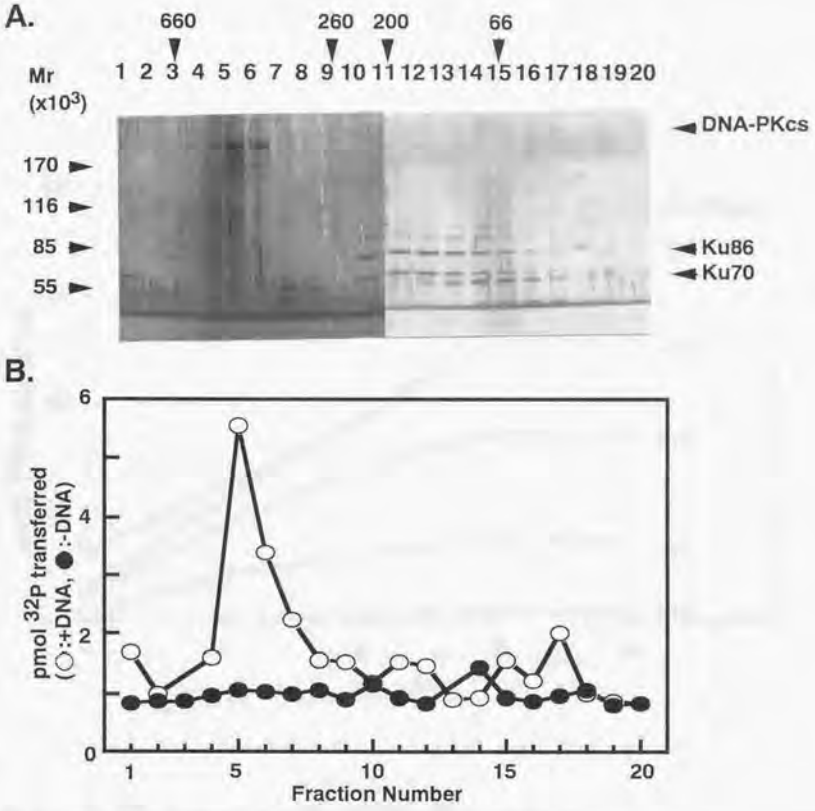

Figure 5. SDS-PAGE patterns and kinase activity of separated DNA-PKcs and Ku subunits.
A, SDS-PAGE patterns of 10μl aliquot from each fraction after glycerol gradient ultracentrifugation, visualized by silver staining. The positions of standard proteins with molecular masses are indicated at the top. B, The kinase activity of 2μl aliquot from each fraction was assayed using synthetic peptide as a substrate in the presence (○) or absence (●) of 20ng/μl of sonicated salmon sperm DNA at 37°C for 10 min.

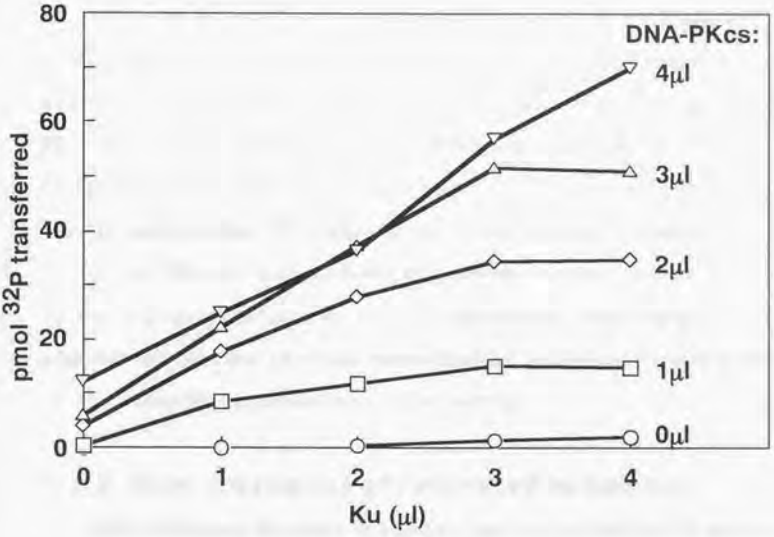

Figure 6. The kinase activity of DNA-PKcs alone or combined with Ku subunits.
Zero to four μl of DNA-PKcs (fraction 5 of Figure 5) alone or combined with 0 to 4 μl of Ku (fraction 12 of Figure 5) was assayed using synthetic peptide as a substrate at 37°C for 10 min with 20ng/μl of sonicated salmon sperm DNA.

3.2. Hyperthermic lability of Ku subunits in DNA-PK.

3.2.1. Heat treatment of DNA-PK holoenzyme.

In order to examine the hyperthermic stability of DNA-PK, protein kinase activity was determined after preincubation of enzyme aliquots either at 37°C or at 44°C. As shown in Fig.7, DNA-PK activity was lost rapidly by incubation at 44°C, whereas DNA-PK incubated at 37°C maintaines the similar level of activity. These results indicate that DNA-PK was sensitive to hyperthermia.

Since DNA-PK is considered the critical enzyme in repair and/or signal transduction of radiation-induced DSB, it might be a possible mechanism that radiosensitization by hyperthermia might be mediated through lability of this enzyme.

3.2.2. Heat treatment of separated subunits.

DNA-PKcs and Ku were separately preincubated for 30 min at either 37°C or at 44°C. Then they were combined and assayed for protein kinase activity. The kinase activity of 37°C-incubated DNA-PKcs mixed with 44°C-incubated Ku ranged from 25 to 55%, depending on purification preparation, of the control, i.e., 37°C-incubated DNA-PKcs combined with 37°C-incubated Ku (Fig.8 lanes 2 and 1), whereas 44°C-incubated DNA-PKcs plus 37°C-incubated Ku exhibited similar activity to that of the control (Fig.8, lanes 3 and 1). The kinase activity of 37°C-incubated DNA-PKcs plus 44°C-incubated Ku and that of 44°C-incubated DNA-PKcs plus 44°C-incubated Ku were similar (Fig.8. lanes 2 and 4). These results indicate that Ku, but not DNA-PKcs, was sensitive to 44°C treatment

and was responsible for hyperthermic instability of DNA-PK holoenzyme.

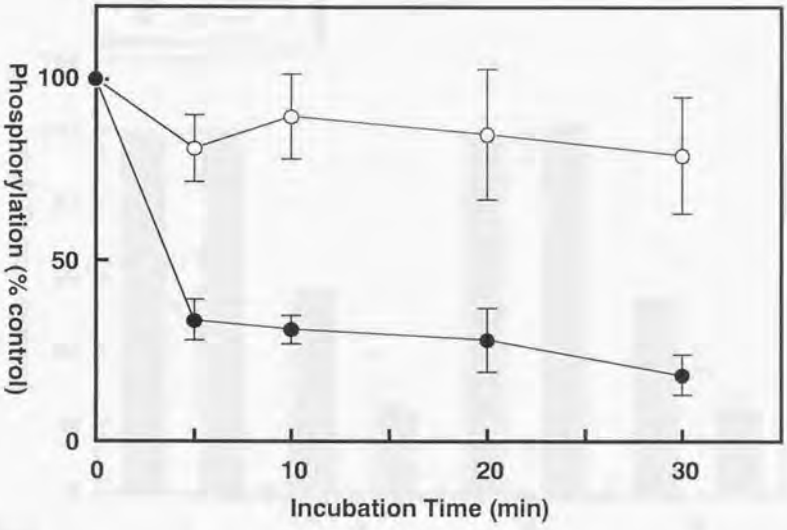

Figure 7. DNA-PK holoenzyme activity after incubation either at 37 °C or at 44 °C.
Purified DNA-PK holoenzyme was incubated either at 37°C (○) or at 44°C (●) for 5, 10, 20 or 30 min and 0.5μl aliquots were assayed for protein kinase activity using synthetic peptide as a substrate at 37°C for 10 min. Activity is expressed as the percentage of control without incubation and standard deviations of three experiments are shown with error bars.

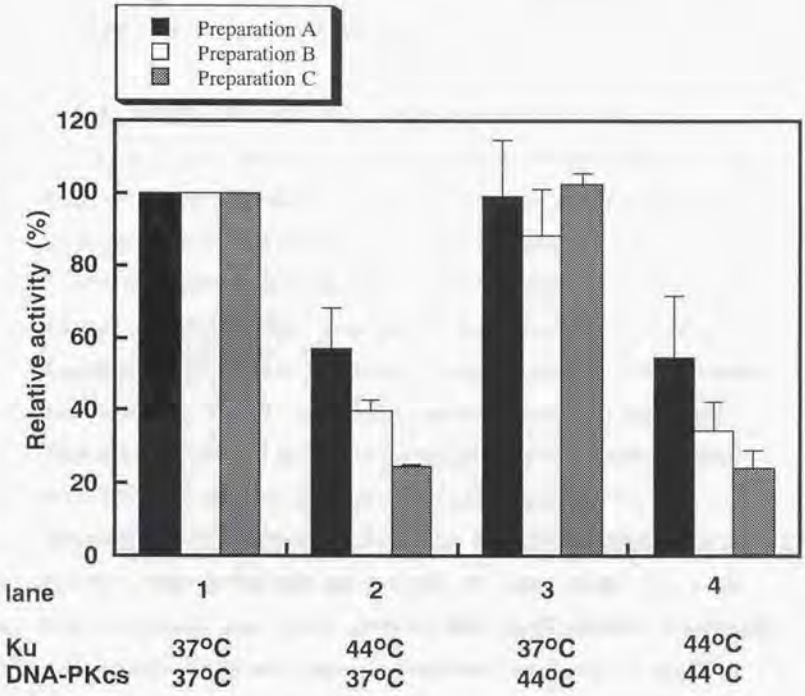

Figure 8. DNA-PK activity after heat treatment of separated subunits.
DNA-PKcs (fraction 5 of Figure 5) and Ku (fraction 12 of Figure 5) were incubated separately either at 37°C or at 44°C for 30 min. Two to four μl each of p470 and Ku were then combined and assayed for protein kinase activity at 37°C for 10 min. Activity is expressed as the percentage of control, i.e., 37°C-incubated DNA-PKcs plus 37°C-incubated Ku (lane 1), with standard deviations shown by error bars among two or three independent assays. Three independent purification preparations of DNA-PK (A, B and C) were separated and tested respectively.

3.3. Heat-stableness of DNA-PK and small hyperthermic radiosensitization in human cells compared to rodent cells.

3.3.1. DNA-PK activity of heat-treated cells.

To examine the hyperthermic stability of DNA-PK inside cells, eight cell lines were heat-treated at 44°C and assayed for DNA-PK activity in crude cell extracts. For this experiment, I modified "pull-down assay" by Finnie et al. (84) and devised a new protocol, here termed "electrophoretic resolution protocol". According to Finnie et al. (84), crude cell extract was mixed with DNA-cellulose and centrifuged prior to kinase reaction in order to concentrate DNA-PK on DNA and to remove most protein kinases other than DNA-PK, most protein phosphatases and possible DNA-PK inhibitor(s). Furthermore, to separate phosphorylation of substrate peptide from that of cellular protein, the reaction mixture after kinase reaction was fractionated by SDS-PAGE, instead of spotting onto phosphocellulose paper as described by Finnie et al. (84).

DNA-PK activity in rodent cells, V79, FSA and FM3A, was decreased by hyperthermia within 60 min (Fig.9 and Fig.11A), but not in human cells, MOLT-4, U937, HL60, MKN-45 and A7 (Fig.10 and Fig.11A). These human cells retained more than 40% of DNA-PK activity even after longer heat treatment, i.e. for 120 or 180 min (Fig.10 and Fig.11B). These results suggest that DNA-PK might be more stable to hyperthermia in human cells than in rodent cells.

Fig.12 shows the clonogenic survival of cell lines after 44°C-treatment. Two human cells, MKN-45 and A7 were much more resistant to hyperthermia than rodent cells, whereas MOLT-4, U937 and HL60 showed comparable hyperthermia sensitivity to rodent

cells. Thus DNA-PK might be generally heat-stable in human cells regardless of a large difference in hyperthermia sensitivity.

3.3.2. X-ray sensitivity of heat-treated cells.

Regarding the heat-stableness of DNA-PK in human cells, human cells might be refractory to hyperthermic radiosensitization if hyperthermic radiosensitization might be mediated through hyperthermic lability DNA-PK. To test this, rodent V79 and human MOLT-4 and A7 cells were assayed for chronogenic survival after hyperthermia followed by X-ray irradiation. Though radiosensitization by hyperthermia was observed in all three cells, thermal enhancement ratio (TER) was smaller in human MOLT-4 and A7 cells than in rodent V79 cells (Fig.13, Table4). These results indicate that human cells, compared to rodent cells, are refractory to hyperthermic radiosensitization possibly due to heat-stableness of DNA-PK.

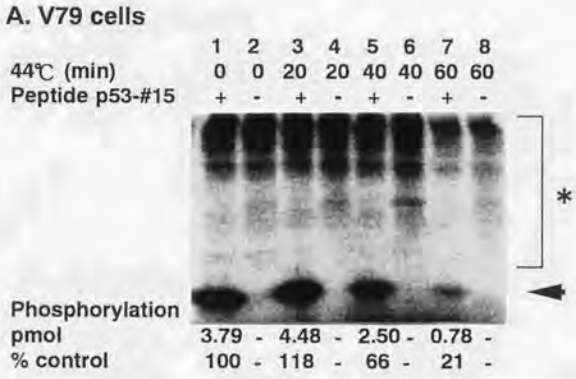

Figure 9. DNA-PK activity of hyperthermia-treated rodent cells.
Crude extract of cells after incubation at 44°C for indicated time was prepared and aliquot containing 50μg protein was used for kinase assay by electrophoretic resolution protocol. The kinase reaction mixture was separated through SDS-PAGE followed by analysis of phosphor image using BAS2000. Quantity of phosphorylated products are shown at the bottom. Arrow heads and asterisks indicate spots of phosphorylated peptide p53-#15 or endogeneous proteins, respectively.
A, V79 cells.

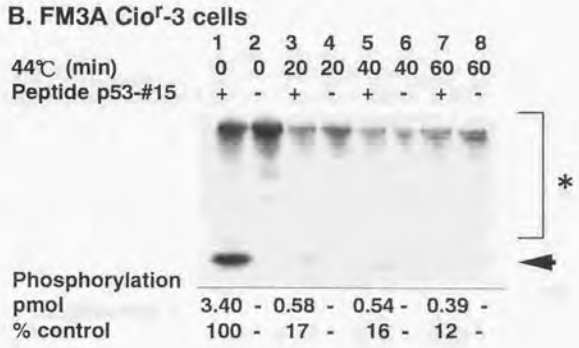

Figure 9. DNA-PK activity of hyperthermia-treated rodent cells.
B, FM3A Cio^r-3 cells.

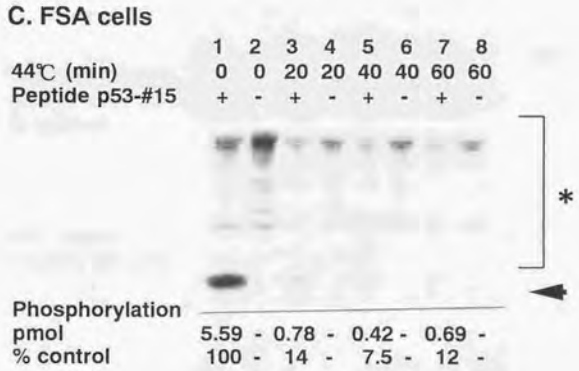

Figure 9. DNA-PK activity of hyperthermia-treated rodent cells.
C, FSA cells.

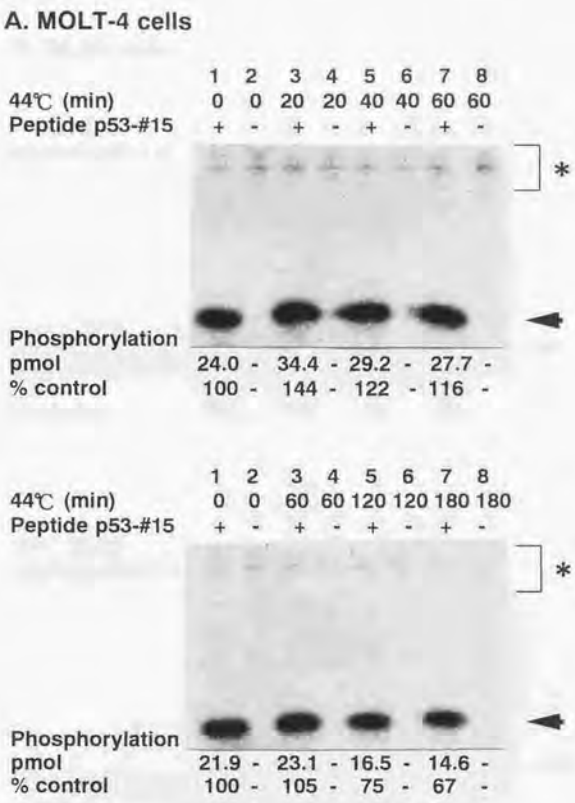

Figure 10. DNA-PK activity of hyperthermia-treated human cells.
Experiments were carried out as described in Figure 9 legend on page 39 except that crude cell extract containing 15μg protein, instead of 50μg, was used for kinase assay. Quantity of phosphorylated products are shown at the bottom. Arrow heads and asterisks indicate spots of phosphorylated peptide p53-#15 or endogeneous proteins, respectively.
A, MOLT-4 cells.
(Upper) 0 to 60 min of 44°C-treatment.
(Lower) 0 to 180 min of 44°C-treatment.

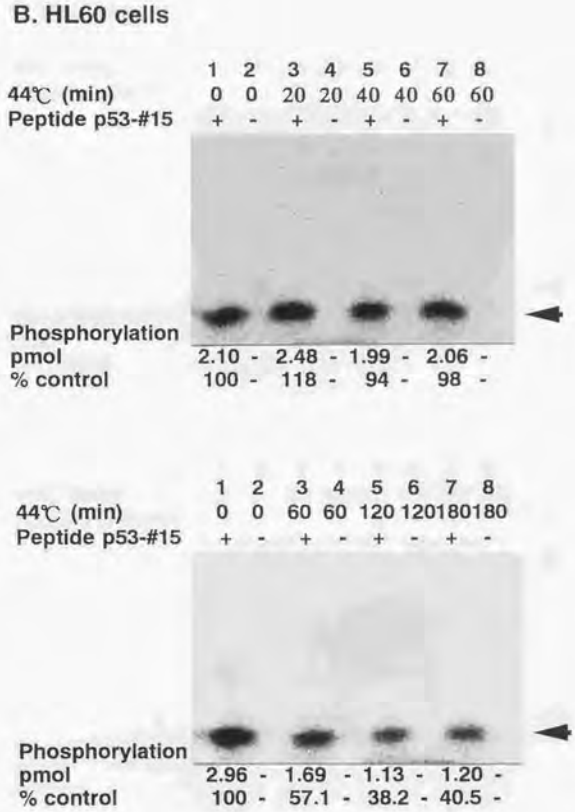

Figure 10. DNA-PK activity of hyperthermia-treated human cells.
B, HL60 cells.
(Upper) 0 to 60 min of 44°C-treatment.
(Lower) 0 to 180 min 44°C-treatment.

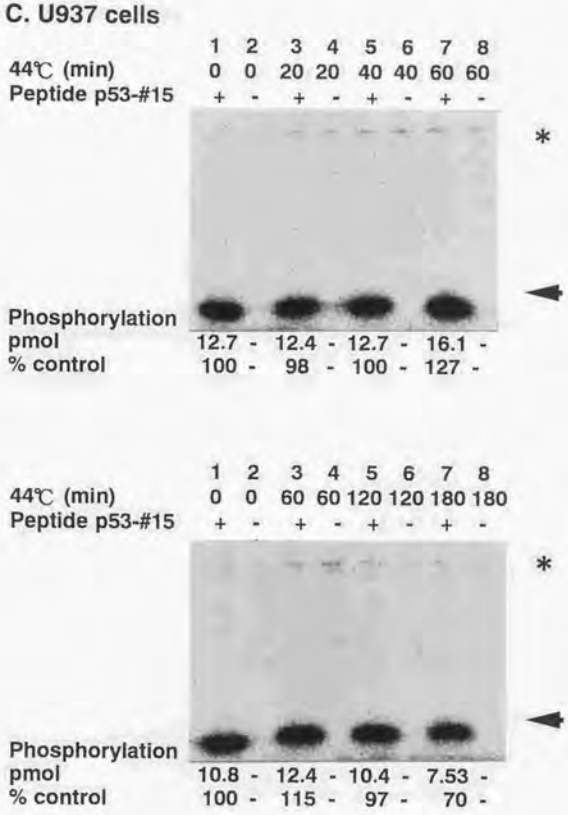

Figure 10. DNA-PK activity of hyperthermia-treated human cells.
C, U937 cells.
(Upper) 0 to 60 min of 44°C-treatment.
(Lower) 0 to 180 min 44°C-treatment.

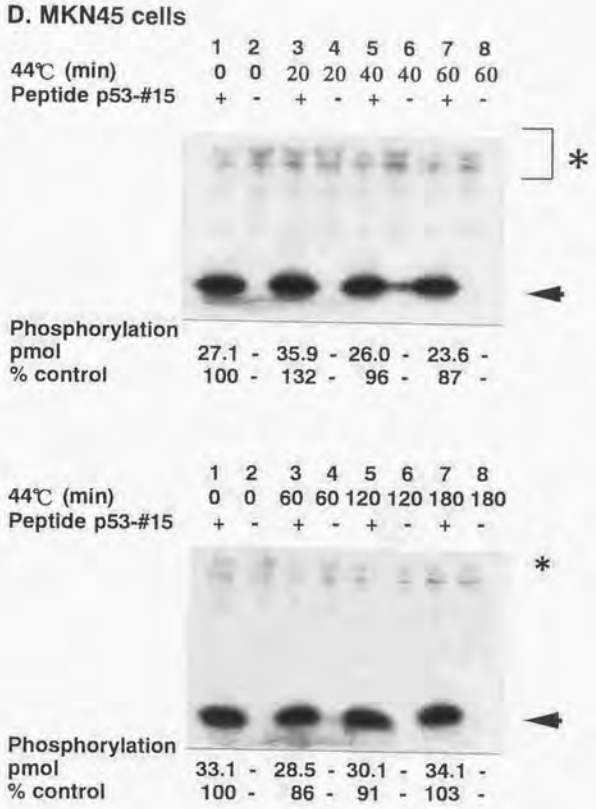

Figure 10. DNA-PK activity of hyperthermia-treated human cells.
D, MKN-45 cells.
(Upper) 0 to 60 min of 44°C-treatment.
(Lower) 0 to 180 min 44°C-treatment.

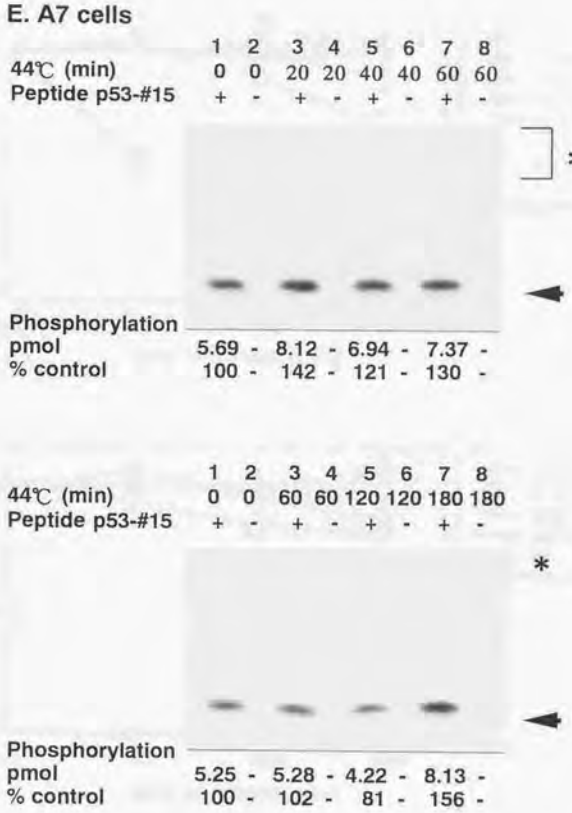

Figure 10. DNA-PK activity of hyperthermia-treated human cells.
E, A7 cells.
(Upper) 0 to 60 min of 44°C-treatment.
(Lower) 0 to 180 min 44°C-treatment.

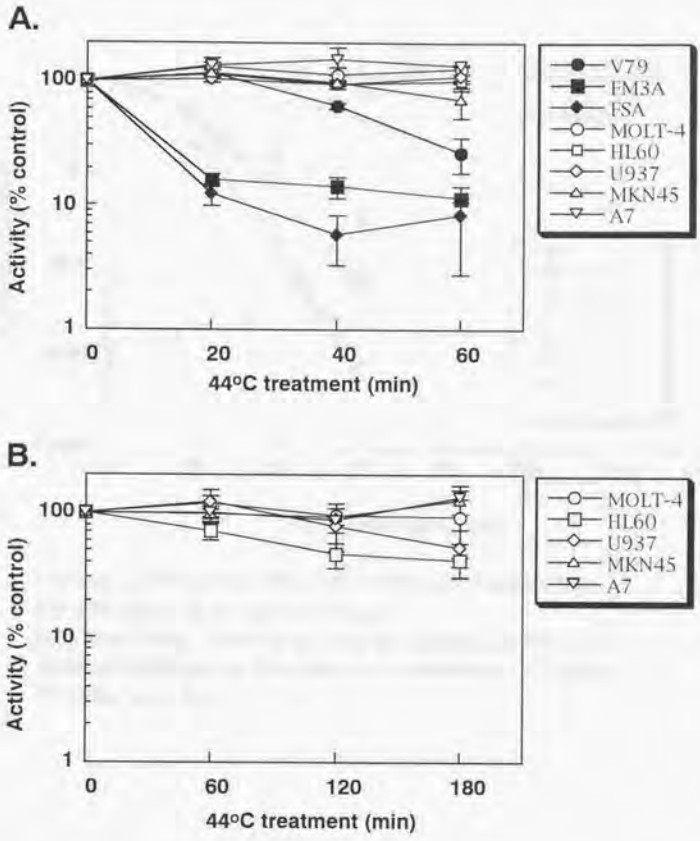

Figure 11. Summary of Figure 9 and 10.
DNA-PK activity of human and rodent cells after 44°C-hyperthermia for 0 to 60 min (A) or for 0 to 180 min (B) were determined as shown in Fig.3-1 and Fig.3-2. The kinase activity is shown as the percentage of unheated control, representing the mean of two or more repeated experiments with standard deviations shown by error bars.

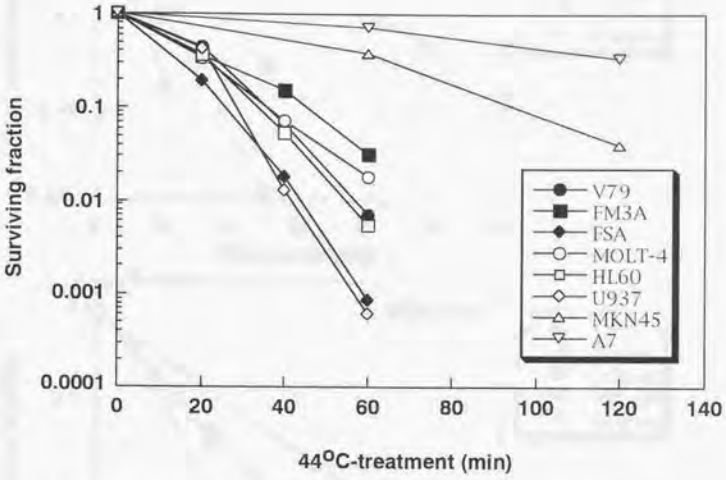

Figure 12. Heating time-cell survival relationship for cell lines used in this study.
The surviving fraction of cells incubated at 44°C for indicated length of time were determined by colony formation assay.

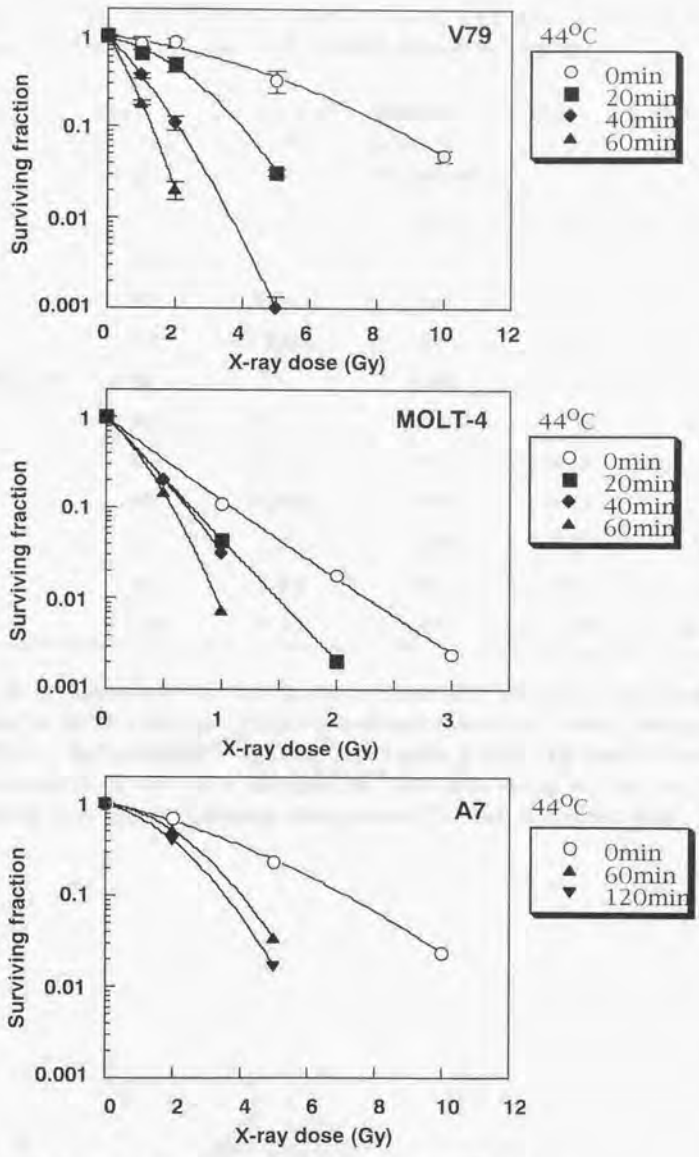

Figure 13. X-ray dose-survival relationship of hyperthermia-treated and untreated cells.

Table 4. Cell survival, DNA-PK activity and X-ray sensitivity of hyperthermia-treated V79, MOLT-4 and A7 cells.

Cell	44oC heating (min)	Surviving fraction	DNA-PK activity (% control)	D_{10}	TER
V79	0	1	100	8.4	1
	20	0.44	114	3.9	2.1
	40	0.067	64	2.1	4.1
	60	0.0071	27	1.3	6.5
MOLT-4	0	1	100	1.1	1
	20	0.34	118	0.73	1.5
	40	0.072	93	0.70	1.6
	60	0.018	91	0.57	2.0
A7	0	1	100	7.2	1
	60	0.72	98	4.0	1.8
	120	0.34	85	3.6	2.0

X-ray dose-survival curves were fitted to linear-quadratic (LQ) model equation, S.F. = $\exp(-\alpha D-\beta D^2)$, by minimal square method as calculated by software "Kaleidagraph". D_{10}, the X-ray dose giving 10% survival, was calculated from the fitted LQ equation. Thermal enhancement ratio (TER) is calculated as D_{10} of unheated cells divided by that of heated cells.

IV. DISCUSSION

4.1. A possible mechanism for hyperthermic radiosensitization mediated through hyperthermic lability of Ku subunits in DNA-PK.

In this study, DNA-PK, an enzyme which is implicated in the repair and/or signal transduction of DNA double-strand break, from human leukemic MOLT-4 cells was purified and characterized. DNA-PK purified from MOLT-4 cells were similar to that from HeLa or Raji cells. The new finding in this study is that DNA-PK is labile at 44°C and that Ku, but not DNA-PKcs, is responsible for the hyperthermic lability. During this study, Burgmann et al. (108) and Ihara et al. (109) showed that DNA-PK in rodent cells was decreased by hyperthermia due to Ku, but not to DNA-PKcs. Additionally, hyperthermic lability of Ku in cell is also indicated by its identification as constitutive heat-shock element binding factor, CHBF, that is downregulated following hyperthermia (110). These results give a new insight into the mechanism of hyperthermic radiosensitization.

As there are lines of evidence (100, 101, 102, 103) indicating that unrepaired DNA double-strand breaks are critical for radiation induced cell killing and that hyperthermia inhibits rejoining of single- or double-strand breaks, potentially lethal damage repair (PLDR) and sublethal damage repair (SLDR), hyperthermic radiosensitization may be considered as a consequence of inactivation of these repair enzymes. Regarding this and S phase sensitivity of cultured cells to heat, hyperthermic sensitivity of DNA polymerases has been extensively studied. Especially DNA polymerase β was sensitive to hyperthermia and its inactivation was correlated to thermal cell killing and radiosensitization (111). Recent studies showed that DNA-PK deficient cells, such as *scid* and

xrs, exhibit high radiosensitivity and impared double-strand break repair (DSBR) and PLDR (20, 112-114), indicating an important role of DNA-PK in the processes of radiation-induced DNA damage repair. Thus, it might be a possible mechanism that hyperthermic radiosensitization is mediated through heat lability of Ku subunits of DNA-PK suppressing radiation damage repair. In support of this idea, there is an evidence that hyperthermic radiosensitization is smaller in Ku86-defective *xrs* cells than parental cells (102).

The repair mechanism and processes of double-strand DNA breaks involving DNA-PK are not well clarified in molecular terms. DNA-PK is supposed to bind to the end of DNA double-strand breaks, to phosphorylate repair enzymes, such as XRCC4/DNA ligase IV complex, controlling their activity and localization and to protect DNA ends from nuclease digestion (39). DNA-PK may have additional roles in signal transduction related to apoptosis or cell cycle regulation, since p53 (11, 12), RPA (13, 14) and c-Myc (51) are also reported as possible substrates. Thus, hyperthermia may inactivate repair enzyme(s) for double-strand breaks, enhance DNA degradation at the DNA break site or modify cellular responses by intervening signal transduction.

However, DNA-PK inactivation might not be solely responsible for hyperthermic radiosensitization, since MOLT-4 and A7 cells were radiosensitized without significant decrease in DNA-PK activity. Additionally, Iliakis et al. showed that hyperthermic radiosensitization is also evident in Ku86-deficient cells, though less evident than in parental cells (102). Additional mechanisms for hyperthermic radiosensitization might be mediated through inactivation of some DNA repair factor other than DNA-PK or preferencial killing by hyperthermia of radioresistant S-phase cells.

4.2. A possible explanation for small hyperthermic radiosensitization in human cells with regard to heat-stableness of DNA-PK.

In the third part of this thesis, I showed that DNA-PK is found more heat-stable in human cells than in rodent cells and hypethermic radiosensitization is smaller in human MOLT-4 or A7 than in rodent V79 cells. Roizin-Towle et al. (115) showed that thermal enhancement ratio (TER) is larger for four tested rodent cells than for eight tested human cells tested. Regarding the critical importance of DNA-PK in the repair or signal transduction of radiation-induced DSBs, smaller TER in human cells than in rodent cells might be due to heat-stableness of DNA-PK activity.

The basis of the heat-stableness of DNA-PK in human cells is currently elusive. One possibility is that human DNA-PK per se is heat-stable. However, this might not be likely, since human DNA-PK purified from MOLT-4 cells is heat-labile. Another possibility is that human cells have some mechanism protecting DNA-PK from hyperthermic inactivation. Regarding this, there are lines of evidence indicating that human cells are generally more heat-resistant than rodent cells (115-119). In this study, MKN-45 and A7, were found much more heat-resistant than rodent cells, while heat-sensitivity of MOLT-4, HL60 and U937 were comparable to rodent cells. Since MOLT-4, HL60 and U937 are lymphatic origin, human cells of this origin might be especially heat-sensitive compared to those of other origin. Nontheless DNA-PK in these cells are similarly heat-stable to heat-resistant MKN-45 and A7 cells, indicating that heat-stableness of DNA-PK is intrinsic to human cells. It is intriguing whether heat-stableness in human cell is

specific to DNA-PK or applicable to other proteins or enzymes. Regarding this, DNA polymerases α and β are shown to be more heat-stable in human glioma cells than in rodent CHO cells (118).

4.3. Perspectives.

Here I showed evidence that DNA-PK is labile at 44°C due to lability of Ku subunits and DNA-PK in human cell is heat-stable compared to that in rodent cells. On the basis of these results, I proposed as a possble mechanism that hyperthermic radiosensitization might be at least partially mediated through the heat lability of DNA-PK and that smaller hyperthermic radiosensitizing effect in human cells compared to rodent cells might be due to heat-stableness of DNA-PK. Though it is well established that hyperthermia inhibits DSB rejoining in rodent cells, it should be compared to that in human cells. Additionally, since DNA-PK is thought to phosphorylate proteins including XRCC4/DNA ligase IV, p53 and RPA, it should be examined whether hyperthermia may inhibit radiation-induced phosphorylation of these proteins and inhibition might be alleviated in human cells compared to rodent cells.

Acknowledgements

A special note of thanks goes to Prof. Norio Suzuki for all his advice, discussion and encoragement throughout the author's entire career as a graduate student.

The author also thanks to Dr. Kazuo Sakai, who trained the author in cell culture experiment during he was an undergraduate student and lead him enter the field of radiobiology.

Thanks also goes to Dr. Hiromu Murofushi, who initiated the author's studies in DNA-PK, for helpful advice and disucussion.

The author expresses thanks to all the members of the Department of Radiation Oncology, Dr. Kazuya Hirano, Dr. Masamitsu Itoh, Ms. Masako Miyata, Ms. Terumi Mizukoshi, Ms. Hiromi Naka, Ms. Kang Yun, Ms. Hong Shu Yin, Mr. Akinori Morita, Mr. Atsushi Enomoto, Ms. Ma Xue Jun, Ms. Noriko Umeda and Ms. Hiromi Inoue for all their advices, discussions, helps and encouragement. Ms. Noriko Umeda is especially acknowledged for sharing part of data. The author also expresses thanks to his friends, especially Mr. Akeshi Morimatsu and Mr. Mitsunobu Hoshino, for encouragement and discussions.

The author thanks Dr. Kazuo Yamamoto for assistance in synthesizing DNA-PK substrate peptide, Dr. Koji Okuhara, Dr. Hirobumi Teraoka and Dr. Fumiaki Watanabe for valuable advice and discussion, Dr. Tsuneyo Mimori, Dr. Koichi Okumura and Shionogi Institute for Medical Sciences for providing antibodies, Dr. Shin-ichi Naritaka for cell lines and discussion and Dr. Makoto Takeuchi and Hayashibara Biomedical Labs Inc. for a large amount of MOLT-4 cells.

The author finally thanks his wife Michiko and his daughter Kaori for their encouragement and pleasantness, which enabled the author to concentrate on his work.

The author, Yoshihisa Matsumoto is a Research Fellow of the Japan Society for the Promotion of Science.

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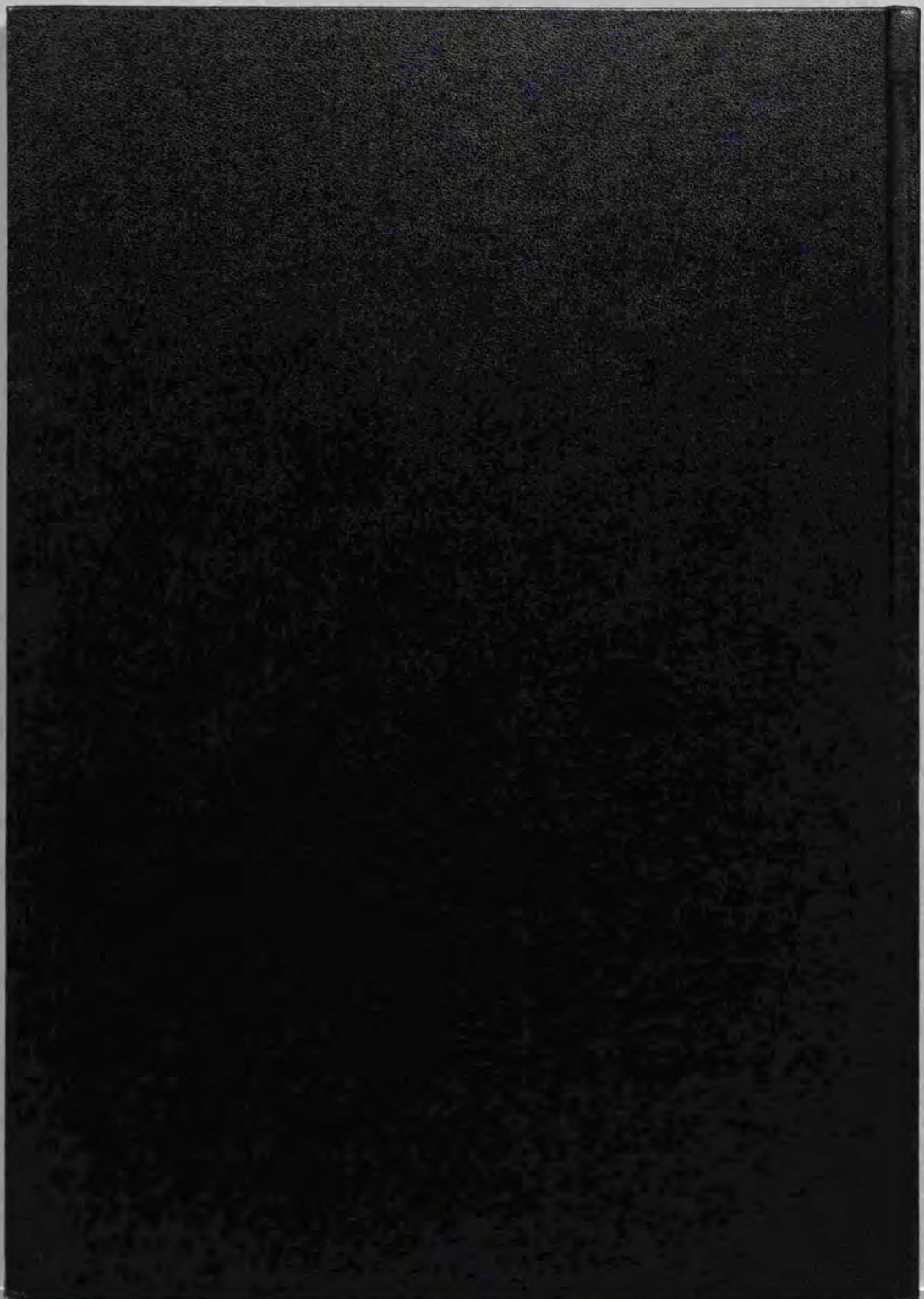
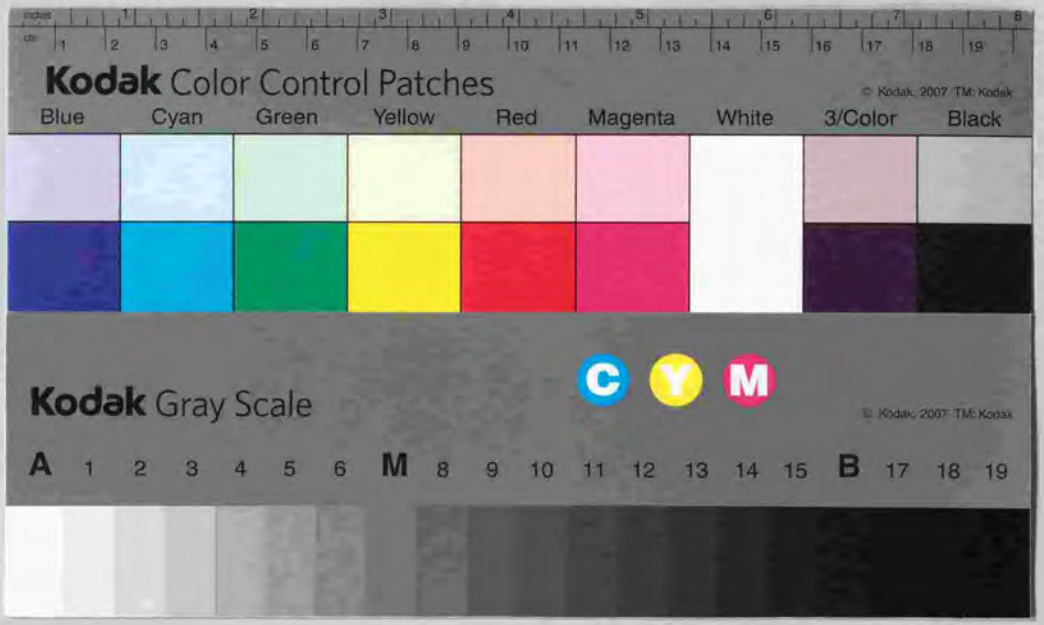
Note

Part of this work is published in Biochemical and Biophysical Research Communications: Matsumoto,Y., Suzuki,N., Sakai,K., Morimatsu,A., Hirano,K., Murofushi,H. (1997). A possible mechanism for hyperthermic radiosensitization mediated through hyperthermic lability of Ku subunits in DNA-dependent protein kinase. *Biochem.Biophys.Res.Commun.* **234**, 568-572.

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