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Prompt Activation of Telomerase by Chemical Carcinogens in Rats Detected with a Modified TRAP Assay

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The maintenance of telomere length is crucial for survival of cells. Telomerase is an RNA-containing reverse transcriptase, which is responsible for elongation of shortened telomeres. Telomerase reactivation has been suggested to be involved in malignant progressions. To study on the involvement of telomerase activation in *in vivo* carcinogenesis, we first modified the original TRAP assay by changing the primer designs and the labeling method of PCR products to an end-labeling method. Second, we investigated the activation of telomerase in different organs after treatments of rats with various chemical carcinogens. Very early after the beginning of the treatment, telomerase activity in the liver, kidney, and lung was increased. In most cases, telomerase activation occurred in the primary or favorite target organs. The present results suggest that telomerase activation occurs promptly when animals are exposed to chemical carcinogens, which may contribute to *in vivo* chemical carcinogenesis.

The ends of vertebrate chromosomes are composed of the specialized structures called telomeres which contain the TTAGGG repeats. Telomeres are involved in stabilizing and protecting the chromosomes.

Normal somatic cells lose telomeric repeats with each cell division, which is called "end replication problem." As a factor to solve this problem, telomerase activity had been foreseen for sometime when Greider et al. (1) first reported in 1985 the detection of telomerase activity by biochemical methods in tetrahymena extracts. Telomerase, a reverse transcriptase contains an RNA component in it and can elongate the shortened telomere DNA. In human, Morin et al. first reported telomerase activity in HeLa cells in 1989 (2).

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Since Kim et al. reported the TRAP assay (3), a variety of cells and tissues including different kinds of cancer cells have been assayed for telomerase activity with this new technique. Telomerase activity was detected in most of cancer cells, but in only some kinds of normal somatic cells such as proliferating lymphocytes and stem cells in regenerating human tissues (4). The elucidation of the regulation mechanisms of telomerase activity appears an important and essential step toward understanding carcinogenesis *in vivo.* Furthermore, telomerase seems one of promising therapeutic targets because most of cancer cells have telomerase activity while most of normal cells do not.

Since the advent of the original TRAP assay (3), many laboratories reported telomerase activities in different kinds of cancers. Most of them, however, measured telomerase activity of already established cancer cells, but not of cells in the midst of the process toward malignant transformation. While the down-regulation of telomerase activity accompanying *in vitro* differentiation of certain kinds of cells such as HL-60 and NB4 cells was studied by many researchers (5, 6), not so many studies on the up-regulation of telomerase activity during carcinogenesis have been reported except some studies such as those on telomerase activation of hyperplastic nodules of the rat liver during hepatocarcinogenesis, of T cells during T cell activation and of human fibroblasts during immortalization (7, 8, 9). We wanted to have insights into the up-regulation of telomerase activity during malignant *in vivo* transformation.

The original TRAP assay (3) appears to have some shortcomings which may be improved such as the internal labeling method and the design of the mismatched primer(see MATERIALS AND METHODS). Our present work has the binary purposes. One is to describe some modifications of the original TRAP assay (3). The other is, with these modifications in hands, to study on the up-regulation of telomerase activity during *in vivo* carcino-

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genesis in rats. The results of the present study suggest that telomerase activation occurs in, and may contribute to, early sages of *in vivo* chemical carcinogenesis.

MATERIALS AND METHODS

Cells and tissue culture. VA13 was obtained from Dr. Koprowski, which is an SV40-immortalized cell line, derived from normal human fibroblast WI-38 (10). YH-1 is a normal human fibroblast strain from which an SV40 -immortalized cell line B-32F was derived (11). PC12 and HL-60 are a rat pheochromocytoma cell line and a human promyelocytic cell line, respectively.

Animals, carcinogen treatment, and partial hepatectomy. Donryu rats weighing 180-200g were divided into 6 groups. A group was fed with a basal diet containing 0.06% 3′-methyl-4-dimethyl-aminoazobenzene(3′-Me-DAB) which has a potent carcinogenic effect on the rat liver. Second and third groups were intraperitoneally given aflatoxin B1 (AFB1, 3 μg or 30 μg/rat/day), everyday. A fourth group was given 1-methyl-1-nitrosourethane(MNUR, 2 mg /kg body weight), intraperitoneally, twice a week for the indicated periods. A fifth group was given drinking water *ad libitum* containing diethylnitrosamine (DEN, 100 ppm). Two Donryu rats were partially hepatectomized and cell extracts were prepared from the regenerating livers 12 and 24 hr after the operation. A group of Wister rats weighing 180-200 g were administered dimethylnitrosamine (DMN, 60 μg/kg body weight) intraperitoneally by one shot. At the indicated times after the beginning of the carcinogen treatments, rats were sacrificed and cell extracts were prepared as described by Kim et al. (3).

To obtain the regenerating liver, two-thirds of the liver were removed surgically by the method of Higgins and Anderson (12).

Telomerase assay. For assaying telomerase activity, we basically followed the original TRAP assay(3), but with some modifications described later. The major points of the modifications are in the primer designs, the PCR conditions and the labeling method of PCR products.

Kim et al. (3) used as the downstream primer the CX primer, which contains the designed mismatches with the TTAGGG repeats. We designed the CX-2 primer (5′-A CTCCCTAACCCTAACCCTAACCC-3′) in place of the CX primer as the downstream primer, which is fully complementary to the 5′-TTAGGG-3′ repeats except the trinucleotide tag ACT at the 5′-end. This modification in the sequence complementarity was intended to increase Tm of our downstream primer, in other words, to increase the priming efficiency of the primer during early cycles of PCR. The 5′-end ACT tag was added to prevent formation of shorter PCR products. We designed the TS-2 primer (5′-ATTGCCAATCCGTCGAGCAGAGTT-3′) as the upstream primer. During the first 25 cycles of PCR, a set of the non-radioactive primers TS-2 and CX-2 was used. To label the PCR products, the original TRAP assay adopted an internal labeling method. We, instead, labeled PCR products by a 5′-end labeling method. We designed the TS-3 primer (5′-GCCAATCCGTCGAGCAGAGTTAGGG-3′) and prelabeled it with 32p at the 5′-end by T4 polynucleotide kinase, and used it only during the last 2 cycles of PCR. We reasoned that with this labeled TS-3 primer we could obtain stricter stoichiometry between the radioactive intensity and the amount of PCR products, because one atom of 32p will strictly represent one molecule of PCR products. The TS-3 primer is designed to have higher Tm than that of TS-2 in order to facilitate the usage of radioactive TS-3 as the favorite primer in the coexistence of cold TS-2 during the last 2 cycles of PCR.

Since the modified TRAP assay was carried out mostly as previously described, here, we describe briefly the procedures of our modified TRAP assay. Samples of cell extracts were prepared as previously reported by Kim et al. (3) and were incubated at 23°C for 30 min to allow telomerase to synthesize extension products. Then, the samples were heated to 85°C for 10 min, added with Taq polymerase and subjected to 25 PCR cycles at 94°C for 1 min, 62°C for 1 min, 60°C for 1 min and 74°C for 1 min, and then, further to the last 2 cycles for labeling PCR products with the 32p-labeled primer TS-3; 94°C for 2 min, 67°C for 1 min, 65°C for 1 min and 74°C for 5 min, and 94°C for 2 min, 67°C for 2 min and 74 ° C for 10 min. The PCR products were subjected to the electrophoretic analysis.

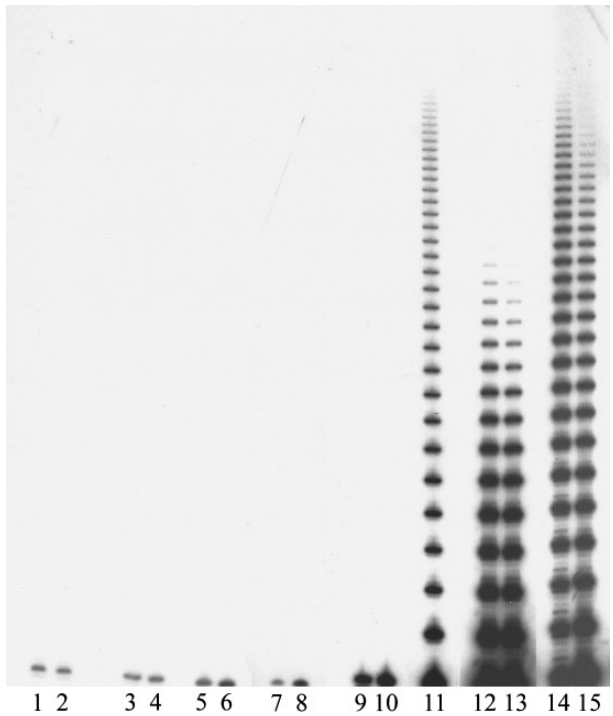

FIG. 1. Detection of telomerase activity by the modified TRAP assay. Cell extracts were prepared from various sources as previously described (3), and two aliquots of each of them containing 2 μg or 5 μg protein were assayed for telomerase activity except lane 11. Lanes 1 and 2; lysis buffer only, 3 and 4; YH-1, 5 and 6; VA13, 7 and 8; the B-32F cell extract preheated, 9 and 10; the B-32F cell extract pretreated with RNase, 11; B-32F (1 μg protein), 12 and 13; PC12, 14 and 15; HL-60.

For RNase pretreatments, 1 μl RNase (10 mg/ml) was added to 100 μl of a reaction mixture and incubated at 36° C for 10 min and then, further incubated at 23°C for 30 min as standard reactions for the primer extension by telomerase. For heat pretreatments cell extracts were preheated at 85°C for 15 min.

Telomerase activity was quantified after electrophoresis by phosphorimaging with BAS 2000 (Fuji Film, Tokyo, Japan). To quantify the intensity of radioactivity of each band on each lane of a polyacrylamide gel, a rectangle of equivalent size and shape was used and we measured only single small telomerase-specific band instead of summing up all bands on each lane. In some cases, after electrophoresis PCR products were visualized by autoradiography and the images were quantitated with a computer program NIH image. Telomerase activity was represented as % activity of the B-32F cell extract (1 μg protein) or control cell extracts. Cell extracts were estimated telomerase positive when a 6 bp ladder was clearly observed compared with a control lane of null cell extract(lysis buffer only) and the ladder was eliminated by RNase and heat pretreatments.

RESULTS AND DISCUSSION

First of all, to estimate the validity of our method, we performed a series of experiments. FIG. 1. shows a

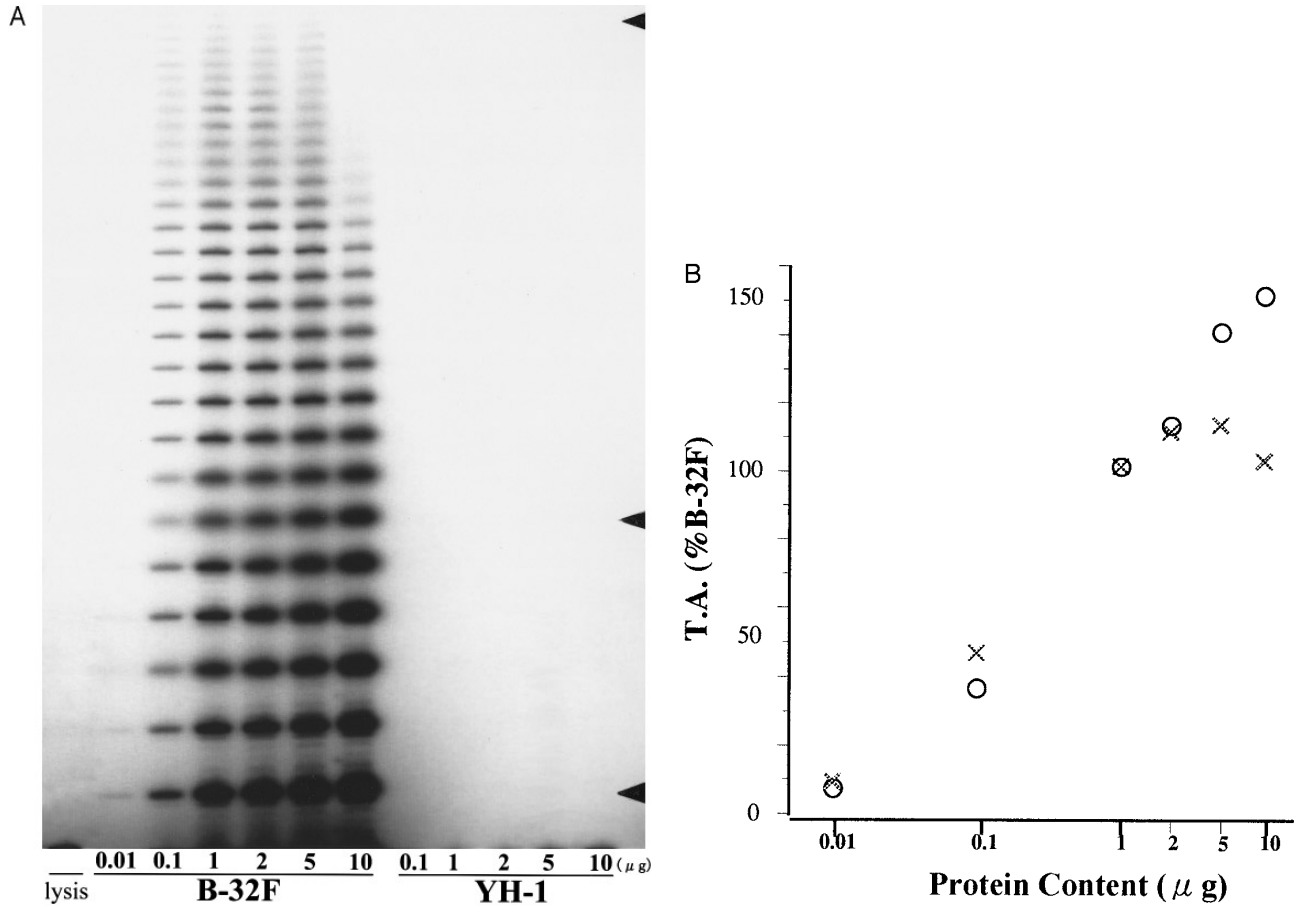

FIG. 2. Dependency of telomerase activity on the protein amount of a cell extract. (A) Different amounts of cell extracts from B-32F and YH-1 cells were assayed for telomerase activity. Numbers on abscissa represent the protein amount in μg per each assay. The leftmost lane (lysis) stands for lysis buffer only. The upper and lower arrowheads indicate the position of the PCR product of 233 nt and the telomerase specific shortest PCR product (53 nt), respectively. (B) The PCR products shown in (A) of the B-32F cell extract were quantitated and plotted against the protein amounts. The ordinate and abscissa represent arbitrary units and the protein amount in μg per each assay reaction on the logarithmic scale, respectively. Crossings; quantitation by summing up all the PCR products. Open circles; quantitation by measuring only one band (83 nt) which is indicated by the middle arrowhead in (A).

result of such an experiment assaying for telomerase activity of some samples by our presently modified TRAP assay. Lane 1 and 2 show null activity of telomerase for lysis buffer only, which means that our present method will not give artificial false positive results. B-32F, a human immortal cell line derived from normal human fibroblasts YH-1, has high telomerase activity (lane 11) as HL-60 and PC-12 cells (lanes 12-15) while VA13 cells and normal YH-1 cells have no activity (lanes 3-6). Lanes 7-10 show that heat and RNase pretreatments of B-32F cell extract abrogated telomerase activity, which means we are really measuring telomerase activity. These results are consistent with earlier reports and indicate our modified TRAP assay is reasonably reliable (5, 13, 14).

To estimate the ability of our modified method to quantitatively assess telomerase activity, we measured telomerase activity of different amounts of the B-32F and YH-1 cell extracts (FIG. 2A). No telomerase activity in YH-1 cells was detected at all in the range of protein amounts tested. Telomerase activities quantitated in FIG. 2A were plotted against the protein amounts of assay reactions (FIG. 2B). We could not get a good positive correlation between the telomerase activities and the amounts of cell extract when the radioactivities of all bands of a ladder were summed up. We found, however, a good positive correlation when the radioactivity of only a single band of telomerase-specific small PCR products was measured and compared (FIG. 2B).

To normalize the efficiency of PCR in each assay, Wright et al. reported the usage of an internal standard (ITAS), which permitted the linearization of the TRAP assay (15). We also tried to develop by ourselves an internal standard similar to ITAS. We could not, however, get the constant amounts of PCR products derived

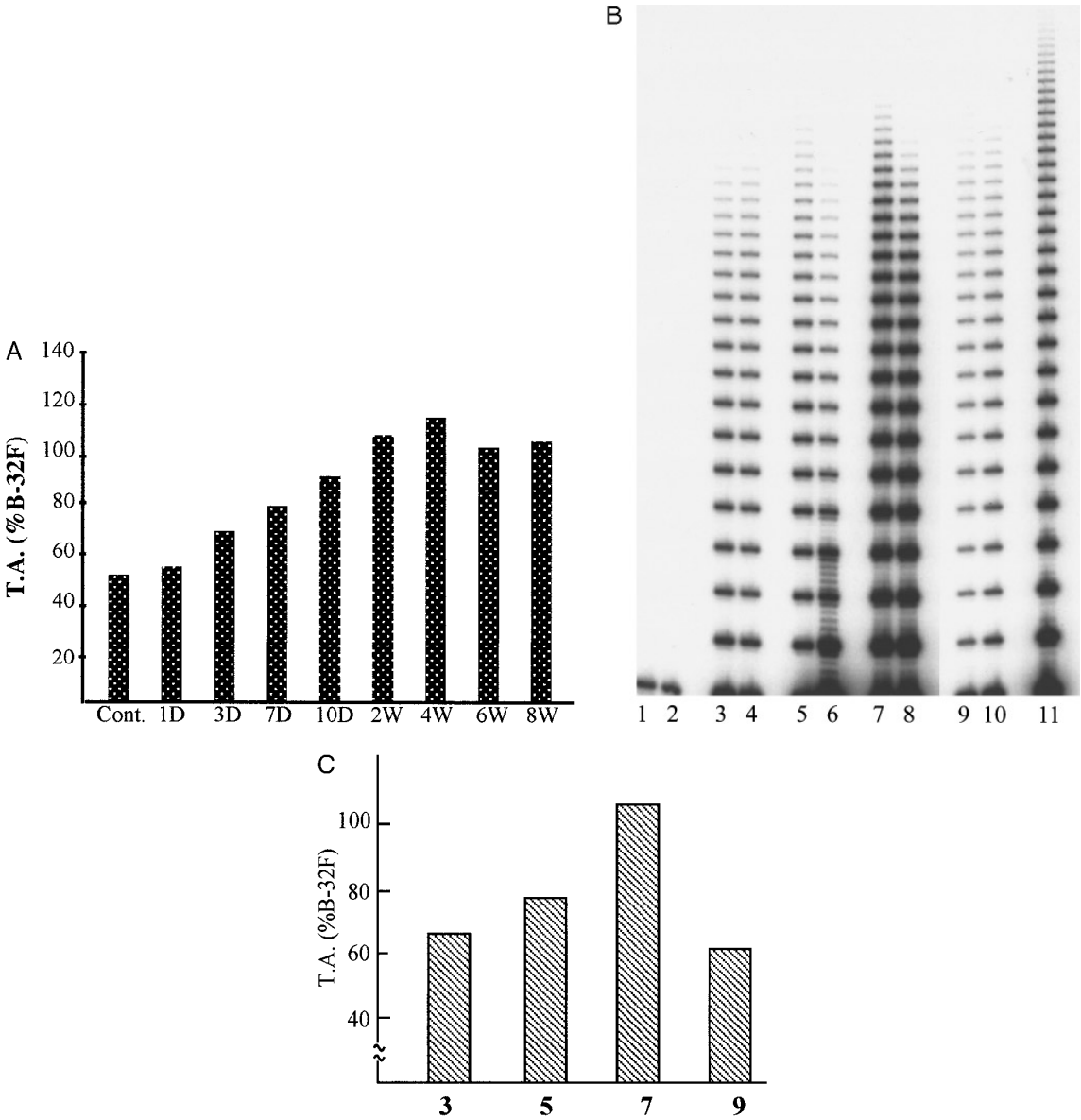

FIG. 3. Telomerase activation during hepatocarcinogenesis by 3′-Me-DAB. (A) Rats were fed with a basal diet containing 0.06% 3′-Me-DAB for the period indicated. D and W on the abscissa stand for day(s) and weeks, respectively. Telomerase activity in liver cell extracts is represented as % activity of the B-32F cell extract (1 μg protein). (B) Rats were given 3′-Me-DAB as in (A) for 4 days or 7 days. For some of the rats, the diet containing the carcinogen was changed back to the normal basal diet with no carcinogen. Lanes 1 and 2; lysis buffer only, lanes 3 and 4; normal rat liver, lanes 5 and 6; 3′-Me-DAB for 4 days, lanes 7 and 8; 3′-Me-DAB for 7 days, lanes 9 and 10; 3′-Me-DAB for 4 days and then the normal basal diet for 4 days, lane 11; B-32F (1 μg protein). In lanes from 3 to 10, a volume of cell extract containing 2 or 5 μg protein was used for each assay. (C) The bands of 83 nt in the lanes 3, 5 , 7 and 9 in (B) were quantitated and represented as % of B-32F telomerase activity (lane 11). The numbers on abscissa correspond to the lane numbers in (B).

from the internal standard, which fluctuated from assay to assay depending on the amount of a cell extract and telomerase activity of the extract. We noticed the tendency that the higher the telomerase activity, the lesser the PCR products derived from the internal standard. It appears that a competition occurs for the primers and Taq polymerase between telomerase products and internal standard molecules when a set of the same two primers are used for both telomerase products and molecules of the internal standard. Therefore, we did not use the internal standard in the present study.

In an attempt to estimate the capability of our modified TRAP assay, we measured telomerase activity of an cell extract from normal fibroblasts of a murine cell line. Our modified TRAP assay could semi-quantitatively detect low telomerase activity while the original TRAP assay could not (data not shown). Taken together

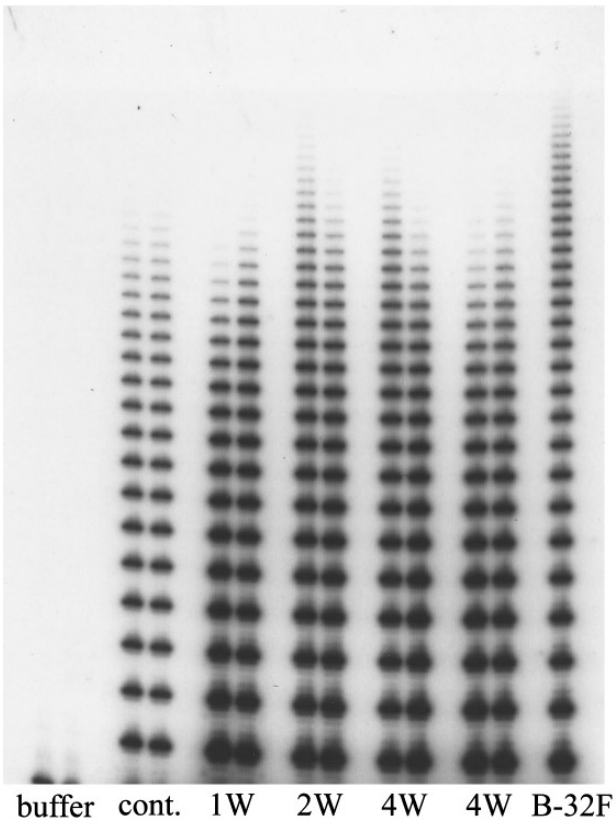

FIG. 4. Telomerase activation during hepatocarcinogenesis by DEN. Rats were given drinking water containing DEN (100 ppm) for the period indicated. Cell extracts were prepared and assayed for telomerase activity as in FIG. 1. buffer; lysis buffer only, Cont.; control, W; week(s). Each of 4W stands for the result of each of 2 independent rats.

with these results, we concluded that our modified TRAP assay is a sensitive, a reasonably semi-quantitative, and hence a reasonably reliable method in a certain range of protein amounts in the assay reactions.

To shed lights into the up-regulation of telomerase activity during *in vivo* carcinogenesis, we planned a series of experiments to administer rats with different kinds of carcinogens and to measure telomerase activity in different organs during the period of *in vivo* carcinogenesis.

FIG. 3. shows a representative result of such experiments. 3′-Me-DAB is one of the most potent hepatocarcinogens for rodents and its primary target organ is the liver (16). Telomerase activity, although slightly, increased already 3 days after the beginning of feeding with 3′-Me-DAB, and further continually increased until 4 weeks (FIG. 3A). Then, it thereafter declined somewhat, but still maintained higher levels than the control value. Three or four rats were sacrificed for each point, but the increase was statistically significant judging from the values and their standard deviations ($P < 0.05$) (data not shown). In an earlier report on 3′-Me-DAB carcinogenesis in rats, foci of nodular hyperplasia appear in the peripheral area after 6 weeks, and from 3 weeks in the earliest case (16). They grow most prominently from the 6th to 9th week, so that the majority of the liver becomes occupied by the lesion (16). We did not, however, in the present study observe any pathological changes including inflammatory and cancerous lesions in the rat liver at least until 8 weeks under the optical microscope(data not shown). Therefore, telomerase activation does not seem to be due to cellular non-specific responses. FIG. 3B and C show that the telomerase activity once elevated due to feeding with 3′-Me-DAB for 4 days returned promptly in 4 days to the control level. The present result in FIG. 3. indicates that telomerase is activated very early in *in vivo* chemical carcinogenesis, the activation is due to the carcinogen, and telomerase activity returned promptly to the control level when the carcinogen was removed. This further suggests that telomerase activation due to chemical carcinogens may contribute to early processes of *in vivo* carcinogenesis.

DEN can induce cancer in various organs such as the lung, the esophagus and the liver in various animals irrespective of animal species and sex, and its favorite target is the liver (17). A representative result of telomerase activation by DEN in the rat liver is shown in FIG. 4. Already one week after the commencement of the administration of DEN, telomerase activity increased to the twice as high level as the control level, and remained so high at least until 4 weeks. Here, again the carcinogen treatment promptly induced telomerase activation in the rat liver.

TABLE 1

Summary of Telomerase Activation by Carcinogens in Different Organs of Rats

Carcinogens	Organ	T.A.(%)
3′-Me-DAB	Liver	282.6
	Kidney	—
DMN	Liver	—
	Kidney	—
AFB1	Liver	159
	Kidney	—
DEN	Liver	208.8
	Kidney	—
MNUR	Liver	—
	Kidney	—
	Lung	157.1

Note. Cell extracts of the liver, kidney, and lung from rats which have been administered various carcinogens for various time lengths up to 8 weeks were assayed for telomerase activity. A horizontal bar (—) represents no telomerase activation detected. Numerals in column T.A.(%) show the maximum telomerase activity detected during the carcinogen treatments expressed as % activity of the control cell extract from an untreated rat.

The results of the present study on telomerase activation by various carcinogens in different organs of rats are summarized in TABLE 1. The potent hepatocarcinogens, 3′-Me-DAB, AFB1 (18) and DEN induced telomerase activation in the liver, that is, the primary or favorite target organ, but not in the kidney under the present experimental conditions. AFB1 was given as a solution in Dimethylsulfoxide (DMSO), and DMSO only induced slight activation of telomerase in the rat liver. The AFB1 treatment, however, induced significantly higher telomerase activity than the control treatment by DMSO only, and the high or low dose administration of AFB1 (3 μg or 30 μg/rat/day) presented the similar results (data not shown). The results from the experiments with these 3 carcinogens showing telomerase activation only in the liver could be ascribed to the unusual sensitivity of the liver which may be extraordinarily more vulnerable to carcinogens than other organs. Telomerase activation, however, was induced in the lung, but not in the liver and kidney by a potent lung carcinogen, MNUR (19). Under the present experimental condition, DMN is to be able to induce cancer in the rat kidney (20). In the present study, telomerase activation was not induced by DMN, which is a potent carcinogen and is more toxic than DEN (TABLE 1). No activation may be due to the administration method; that is, the administration by a single shot of large quantity of DMN which might have suppressed the activation.

A chemical carcinogen can in *vivo* induce different cancers in various different organs depending on the administration method, animal species, the sex and even strains of the same species (21, 22). Under the experimental conditions of the present work, we detected the induced higher telomerase activity in the primary or favorite target organ although not so many organs were studied (TABLE 1). In conclusion, the results of the present study suggest that the organ-specific telomerase activation occurs promptly and may contribute to the organ-specific carcinogenesis.

Chemical carcinogens can induce the formation of DNA adducts, DNA repair and DNA synthesis (21, 22). To see the effect of induction of DNA synthesis on telomerase activation, rats were partially hepatectomized and telomerase activity was measured at 12 and 24 hr after the operation(data not shown). We did not detect any induction of higher telomerase activity than the control level at 12 hr and also even at 24 hr when the DNA synthesis culminates (23). Therefore, telomerase activation by chemical carcinogens can not be ascribed merely to the stimulation of DNA synthesis and repair or cell growth.

Recently, Tsujiuchi et al. reported markedly increased telomerase activity in hyperplastic nodules and hepatocellular carcinomas induced by a choline-deficient L-amino acid-defined (CDAA) diet, but not in background parenchyma in the rat liver, and they claimed that increased telomerase activity may be a biological feature of preneoplastic lesions (7). Their hepatocarcinogenesis model used a CDAA diet without carcinogen application, thereby our results can not be compared directly with their result.

While our present results suggest Telomerase activation in early stages in *in vivo* carcinogenesis, Hiyama et al. reported in a study on human gastric cancer that telomerase is activated as a late event in gastric cancer progression (24). Their conclusion was obtained from assaying clinical specimens of already established cancer. The two reports, therefore, cannot be directly compared.

Recently, Blasco et al. generated mice deleted for the gene encoding the telomerase RNA component and reported that telomerase is essential for telomere length maintenance but is not required for establishment of cell lines, oncogenic transformation, or tumor formation in mice (25). They clearly showed that murine cells have some way(s) to maintain the integrity of chromosomes without telomerase. Their results, however, will not exclude the possibility of some contribution of telomerase activation to early stages of *in vivo* carcinogenesis.

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