TABLE 1
Antibody Level and Positivity among Atomic Bomb Survivors

	Radiation dose (mGy)		
	0	1–999	≧1000
Number of individuals[a]	1,481	1,368	627
Mean age (years)	71.6	72.1	70.8
Mean dose (mGy)	0	315.8	1524.9
Male (%)	32.1	28.6	37.8
Mean BMI (SE)	23.0 (0.09)	23.1 (0.1)	22.4 (0.1)
Current smoker (%)	14.3	14.2	13.1
Antibody level[b]			
Chlamydia pneumoniae, IgG			
Median level (U)	2.31	2.19	2.00
Mean level (SE) (U)	2.69 (0.036)	2.59 (0.052)	2.49 (0.081)
Positivity (%)	77.7	75.4	71.1
Chlamydia pneumoniae, IgA			
Median level (U)	1.63	1.54	1.41
Mean level (SE) (U)	1.94 (0.036)	1.87 (0.038)	1.79 (0.055)
Positivity (%)	67.9	66.1	63.0
Helicobacter pylori, IgG			
Median level (U)	41.1	38.2	45.2
Mean level (SE) (U)	48.2 (1.1)	46.8 (1.0)	49.9 (1.7)
Positivity (%)	62.7	60.1	63.0
Cytomegalovirus, IgG			
Median level (U)	47.8	51.7	47.4
Mean level (SE) (U)	61.9 (1.8)	62.4 (1.8)	62.0 (2.6)
Positivity (%)	99.2	99.6	99.2
CRP level			
Median level (mg/dl)	0.062	0.066	0.062
Mean level (SE) (mg/dl)	0.11 (0.004)	0.12 (0.004)	0.12 (0.006)

[a] For the measurement of cytomegalovirus IgG, the numbers of subjects were: 0 Gy: 858, 1–999 mGy: 816, and ≧1000 mGy: 375.

[b] Antibody level is presented as median and mean values for quantitative measurements. Positivity is the proportion of individuals whose antibody level was defined as positive, as described in the Materials and Methods, for each dose category.

tibody to *Chlamydia pneumoniae* was significantly and negatively associated with radiation dose. The level of IgA antibody to *Chlamydia pneumoniae* was significantly and positively associated with male gender, older age and cigarette smoking and negatively associated with radiation dose (Table 2). This antibody level was significantly higher in subjects from Nagasaki than in those from Hiroshima (Table 2). The distribution of levels of IgG and IgA antibody to *Chlamydia pneumoniae* in relation to radiation dose is presented in Figs. 1 and 2, respectively. No significant

TABLE 2
Association of Levels of IgG and IgA Antibodies to *Chlamydia pneumoniae* with Radiation Dose (multiple regressions)[a]

	Chlamydia pneumoniae					
	IgG			IgA		
Factors (unit)	Change (%)	95% CI	*P*	Change (%)	95% CI	*P*
Dose (1,000 mGy)	−14.2	−19.7, −8.3	<0.001	−4.9	−8.9, −0.1	0.021
Age (10 years)	29.5	21.5, 38.0	<0.001	18.3	13.6, 23.3	<0.001
Gender (Male: 0, Female: 1)	−34.2	−41.7, −25.4	<0.001	−22.9	−28.6, −11.4	<0.001
Smoking (20 cigarettes/day)	20.1	1.9, 41.6	0.029	19.3	7.3, 32.7	0.001
City (Hiroshima: 0, Nagasaki: 1)	3.8	−6.8, 15.5	0.50	8.9	1.6, 16.7	0.016

[a] Association was examined by linear regression analysis, adjusting for each factor. Values are indicated as percentage change of the antibody level per unit (in parentheses) increase for each factor. Representative antibody level (intercept) (95% CI) for nonirradiated and nonsmoking males at 70 years of age in Hiroshima was calculated to be 2.06 (1.83, 2.32) U for IgG and 1.52 (1.41, 1.65) U for IgA. CI, confidence interval.

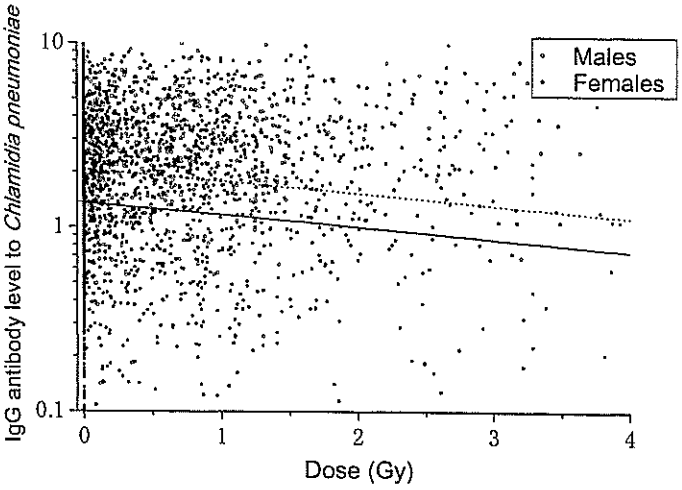

FIG. 1. Scatter diagram of IgG antibody level and radiation dose with regression lines fitted for nonsmoking male (dotted line) and female (solid line) at age 70 years in Hiroshima. Multiple R^2 value for the regression was 0.037.

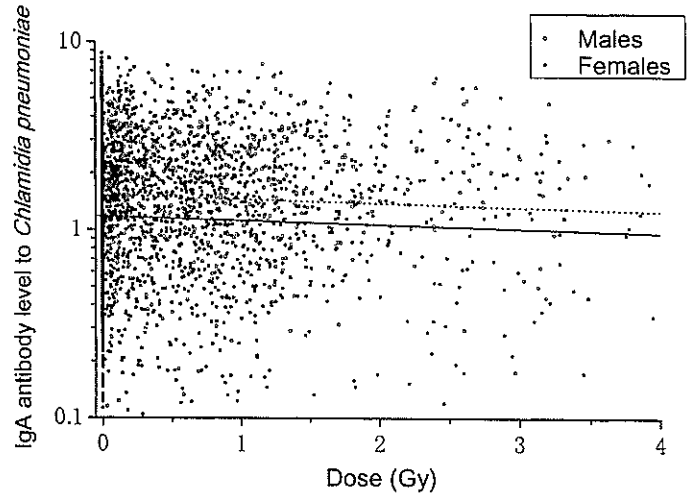

FIG. 2. Scatter diagram of IgA-antibody level and radiation dose with regression lines fitted for nonsmoking male (dotted line) and female (solid line) at age 70 years in Hiroshima. Multiple R^2 value for the regression was 0.035.

dose effect was observed for other antibody levels. The level of IgG antibody to *Helicobacter pylori* decreased with age and was not significantly associated with either cigarette smoking or radiation dose (Table 3). The level of IgG antibody to cytomegalovirus was significantly associated with female gender, older age and cigarette smoking but not with radiation dose (Table 3). In these analyses for association of antibody level with radiation dose, interactions between age, gender, smoking and radiation dose were examined, but no significant interactions were observed.

When antibody level was classified as either positive or negative, association with radiation dose was similar. Thus a negative association was observed between radiation dose and positive response to *Chlamydia pneumoniae* in both the IgG and IgA antibody classes (data not shown). Radiation dose was not significantly associated with positive IgG antibody response to *Helicobacter pylori* or cytomegalovirus (data not shown).

To investigate the biological significance of the decreased antibody level to *Chlamydia pneumoniae* in radiation-exposed survivors, the serum level of CRP, a sensitive marker of inflammation, was examined. In this analysis, samples with CRP levels greater than 1.0 mg/dl were excluded from analysis to avoid confounding by acute infectious disease and systemic inflammatory disease samples. Thus a total of 3,155 samples were analyzed. CRP level was positively associated with radiation dose, age, male gender, smoking and BMI (Table 4). CRP level also differed between Hiroshima and Nagasaki (Table 4). The distribution of the CRP level in relation to radiation dose is presented in Fig. 3.

After adjustment for the factors presented in Table 4, the CRP level was positively and significantly associated with the level of IgA antibody to *Chlamydia pneumoniae* (3.7% increase per 1 U increase in antibody level, $P = 0.006$) but only marginally with the level of IgG antibody to *Chlamydia pneumoniae* ($P = 0.057$) (Table 5). In these analyses, association of CRP level with radiation dose did not change substantially from the results presented in Table 4 [percentage increase in CRP level per 1,000 mGy was 7.0% ($P = 0.004$) and 7.1% ($P = 0.003$) when IgG and IgA anti-

TABLE 3
Association of Levels of IgG Antibody to *Helicobacter pylori* and Cytomegalovirus with Radiation Dose (multiple regressions)[a]

Factors (unit)	*Helicobacter pylori* IgG			Cytomegalovirus IgG		
	Change (%)	95% CI	*P*	Change (%)	95% CI	*P*
Dose (1,000 mGy)	8.9	−10.2, 32.0	0.39	1.9	−3.2, 7.1	0.46
Age (10 years)	−20.6	−34.7, −3.5	0.021	18.1	12.6, 23.8	<0.001
Gender (Male: 0, Female: 1)	−60.4	−72.9, −11.4	<0.001	37.9	26.0, 50.9	<0.001
Smoking (20 cigarettes/day)	8.8	−31.7, 73.2	>0.5	31.4	16.5, 48.2	<0.001
City (Hiroshima: 0, Nagasaki: 1)	19.1	−12.7, 62.4	0.271	2.1	−5.9, 10.8	>0.5

[a] Association was examined by linear regression analysis, adjusting for each factor. Values are percentage change of the antibody level per unit (in parentheses) increase of each factor. Representative antibody level (intercept) (95% CI) for nonirradiated and nonsmoking males at 70 years of age in Hiroshima was calculated to be 58.8 (42.1, 82.1) U for *Helicobacter pylori* IgG and 32.8 (30.0, 35.9) U for cytomegalovirus IgG. CI, confidence interval.

TABLE 4
Association of CRP Level with Radiation Dose (multiple regressions)[a]

Factors (unit)	Change (%)	95% CI	*P*
Dose (1,000 mGy)	6.9	2.1, 11.9	0.004
Age (10 years)	20.4	15.0, 26.1	<0.001
Gender (Male: 0, Female: 1)	−18.8	−25.1, −11.9	<0.001
Smoking (20 cigarettes/day)	27.5	14.1, 42.4	<0.001
BMI (1.0)	7.7	6.6, 8.8	<0.001
City (Hiroshima: 0, Nagasaki: 1)	−12.4	−18.5, −5.7	<0.001

[a] Association was examined by linear regression analysis, adjusting for each factor. Values represent the percentage change of CRP level per unit (in parentheses) increase of each factor. Representative CRP level (intercept) (95% CI) for nonirradiated and nonsmoking males at 70 years of age in Hiroshima was calculated to be 0.072 (0.67, 0.79) mg/dl. BMI, body mass index. CI, confidence interval.

body levels were included in the model, respectively]. In addition, when CRP was included in the adjustment factors for analysis of the relationship between antibody level and radiation dose, the results were similar to those presented in Table 2. Thus a 15.3% decrease in antibody level per gray was observed for IgG (when CRP was not included, a 14.2% decrease was observed, as indicated in Table 2) and a 5.1% decrease for IgA (when CRP was not included, a 4.9% decrease, as indicated in Table 2).

The response of CRP to the *Chlamydia pneumoniae* antibody level did not interact formally with radiation dose (significance level of the interaction between radiation dose and antibody level: $P = 0.36$ for IgG and $P \geq 0.5$ for IgA). To further investigate the decreased antibody levels in radiation-exposed survivors, however, the relationship between CRP level and antibody levels was examined in different dose categories. In this analysis, a significant association of CRP level with IgA antibody level was noted in the high-dose group (≥1000 mGy) but not in the 0-Gy or 1–999-mGy groups (Table 5). The percentage increase in CRP levels per unit of antibody in the high-dose group was approximately twice that in the nonirradiated and intermediate-dose groups (Table 5), although the difference between the groups was not statistically significant. The relationship between CRP level and the level of IgG antibody to *Chlamydia pneumoniae* was also significant in the high-dose group but not in the 0-Gy or 1–999-mGy groups (Table 5).

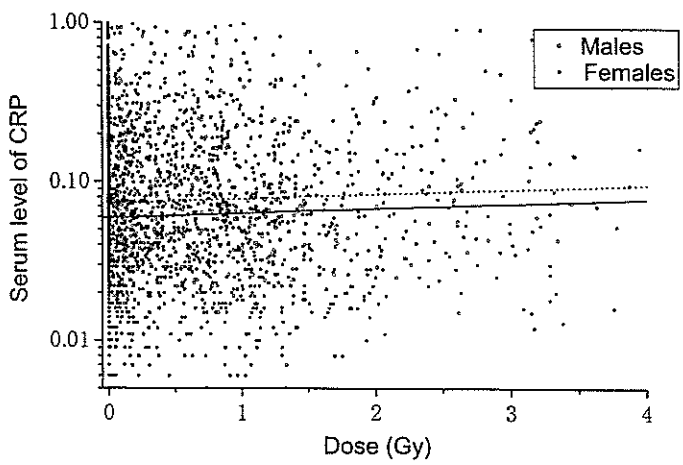

FIG. 3. Scatter diagram of CRP level and radiation dose with regression lines fitted for nonsmoking male (dotted line) and female (solid line) with BMI of 23.0 kg/m² at age 70 years in Hiroshima. Multiple R^2 value for the regression was 0.086.

DISCUSSION

In this cross-sectional study of A-bomb survivors, we observed a radiation dose-associated decrease in the level of antibody to *Chlamydia pneumoniae*. The effects of gender and smoking on level of antibodies to the three pathogens observed in the present study were similar to those reported previously (*23–25*). The limitations of our study may be as follows. Since more than half of the original cohort had died before the start of this study, the possibility cannot be excluded that persons with higher antibody levels might have been preferentially lost from the group of subjects with higher radiation doses. It is also possible that some persons could not participate in the study because of

TABLE 5
Association of CRP Level with Antibody Level to *Chlamydia pneumoniae* Stratified by Radiation Dose[a]

	CRP level			
	No. of subjects	Change (%)	95% CI	*P*
Chlamydia pneumoniae IgG				
Total samples	3,155	1.8	−0.01, 3.7	0.057
0 mGy	1,355	1.2	−1.0, 3.5	0.287
1–999 mGy	1,239	1.5	−0.8, 3.9	0.201
≥1,000 mGy	561	3.5	0.5, 6.6	0.024
Chlamydia pneumoniae IgA				
Total samples	3,155	3.7	1.0, 6.5	0.006
0 mGy	1,355	2.9	−1.3, 6.1	0.075
1–999 mGy	1,239	3.3	−0.1, 6.8	0.055
≥1,000 mGy	561	6.5	2.0, 11.2	0.005

[a] Association was examined by multiple regression analysis, adjusting for city, gender, age, body mass index, and smoking. Percentage change in CRP level is presented for one unit increase in antibody level. CI, confidence interval.

illness related to high or low antibody levels. Further, some other unknown factor(s) not adjusted for in the present analysis might have confounded the results.

Biologically, two possibilities exist for the lower antibody response to bacterial infection. One is a decrease in infectious agents; the other is a decrease in immune response to post-bombing bacterial infection. The former seems less likely in A-bomb survivors due to the exacerbation of hygiene conditions by the complete destruction of lifeline and medical systems after the bombings of the two cities. Diminished immune function has been suggested in radiation-exposed survivors. Thus lymphocyte proliferation induced by mitogens, alloantigens and bacterial superantigens (*13*, *16*), the proportion of T lymphocytes, especially CD4-positive helper T lymphocytes (*14*), and the frequency of T lymphocytes producing interleukin 2 (*15*) are all diminished among radiation-exposed survivors. In addition to these nonspecific decreases in immune function, there may be some antigen-specific impairment among A-bomb survivors, as suggested by repertoire analysis examining the usage of divergent variable region genes of T-lymphocyte receptors in the blood (*26*). Since T lymphocytes are essential for antibody production of B cells, impairment in T lymphocytes in the survivors may affect antibody production.

Similarly, diminished immune response has been implicated in other infections in the survivors. It has repeatedly been shown, for example, that prevalence of hepatitis B surface antigen is increased with radiation dose in A-bomb survivors, whereas prevalence of its antibody is not (*17–19*). Although a smooth dose–response relationship was not observed between the prevalence of antibody to hepatitis C virus and radiation dose, prevalence of the antibody was lower in survivors with a positive dose estimate than in those with a dose estimate of 0 (*27*).

The mechanism behind the absence of a significant association between antibody levels to *Helicobacter pylori* and cytomegalovirus with radiation is not clear, but it may be related to the difference in the frequency of recurrent infection by these pathogens. The first infection among the three pathogens occurs early in childhood, and subsequent recurrent infection is common only for *Chlamydia pneumoniae* (*28*). If immune function at the time of *Chlamydia pneumoniae* infection is related to antibody level, diminished immune function due to A-bomb exposure at the time of the recurrent infection might cause decreased antibody response. Naturally, we cannot estimate either the timing of infection or the cause of the antibody level (i.e., past primary infection, re-infection or reactivation). Of interest is that the increased prevalence of hepatitis B surface antigen with radiation dose among A-bomb survivors is restricted to those who received blood transfusions after A-bomb radiation exposure (*19*). Since persistence of hepatitis B virus antigen is related to diminished immune response to the virus, the radiation dose-associated increase in the prevalence of hepatitis B antigen among the survivors who received blood transfusions suggests impaired immune function from exposure to A-bomb radiation.

Although interaction with radiation dose was not formally significant for the association of CRP level with antibody level, we found a tendency for CRP level to be associated with levels of both IgG and IgA antibodies to *Chlamydia pneumoniae* in the high-dose group. This finding may suggest the presence of infection-related inflammation in heavily exposed survivors. Since persistent infection in macrophages is typically found after the acute phase of *Chlamydia pneumoniae* infection ends, association of CRP level with antibody level may reflect an active state of chronic infection.

We also confirmed, with a larger number of subjects, our previous finding of a positive association of CRP level with radiation dose (*29*). This finding could prove to be important because many studies have demonstrated that a mildly elevated CRP level is a risk factor for cardiovascular disease (*8*, *30*). Association of CRP level with male gender, BMI and smoking, which was found in the present study, was also reported previously (*31*). The reason for the city difference in CRP level found in the present study is unclear. Systemic technical error is not likely because all samples were measured in the Hiroshima laboratory. Since the prevalence of diabetes mellitus among A-bomb survivors is lower in Nagasaki than in Hiroshima (*32*), and since diabetes is associated with increased CRP levels (*33*), we adjusted for diabetes in our analysis, but the city difference remained significant (data not shown).

In conclusion, our study suggests that the immune response to *Chlamydia pneumoniae*, a microorganism implicated in cardiovascular pathogenesis, is diminished in radiation-exposed A-bomb survivors. However, inflammatory response to *Chlamydia pneumoniae* may be present, which would reflect an active state of infection in the survivors.

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A Preliminary Study Measuring the Number of T-Cell Receptor-Rearrangement Excision Circles (TRECs) in Peripheral Blood T-Cell Populations of A-Bomb Survivors and Control Populations

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原爆被爆者末梢血T細胞集団および対照集団におけるT-Cell Receptor-Rearrangement Excision Circles (TRECs) の測定に関する予備的研究

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原爆放射線による免疫系の障害を被った被爆者では，半世紀以上経た今日においてもナイーブCD4 およびCD8T細胞の割合の低下が認められている。今回，被爆者におけるナイーブT細胞集団の減少が胸腺でのT細胞産生の低下によるものか検討する目的で，原爆被爆者末梢血T細胞集団におけるT-cell receptor-rearrangement excision circles (TRECs) を測定するリアルタイムPCR法の確立を試みた。研究室内の対照を用いて行ったリアルタイムPCRでは，良好な再現性でTRECのDNA配列を定量的に検出することができた。これまでに測定を完了した445名について，性，年齢，および被ばく線量を変量とした多重回帰解析を行ったところ，CD4 T細胞集団におけるTREC数は女性で有意に多く，男女とも加齢につれ有意に減少した。また，被爆時の年齢が20歳未満の対象者では，被ばく線量の増加とともに低下する傾向 ($p = 0.09$) が示唆された。CD8 T細胞集団においても同様の性差および加齢の影響が認められたが，有意な放射線の影響は観察されなかった。これらの結果は，原爆放射線被ばくによる胸腺でのCD4 T細胞産生の長期低下の可能性を支持する。この仮説を検証するためには，さらに対象者数を拡大して調べる必要がある。

Keywords: TREC, immune system, radiation, aging

Abstract

More than a half century after damage of the immune systems by the radiation from A-bomb, we can still observe significant decreases in the percentages of naïve CD4 and CD8 T cells among the survivors. To investigate whether the observed decreases in the naïve T-cell populations may have resulted from reduction in thymic T-cell production ability of survivors, we established a real-time PCR method to examine the number of T-cell receptor-rearrangement excision circles (TRECs) in peripheral blood CD4 and CD8 T-cell populations. The real-time PCR quantitatively detected TREC sequences with a good reproducibility in human laboratory controls. In the 445 survivors so far been examined, multiple regression analysis indicated that the number of TRECs in the CD4 T-cell fraction was significantly higher in

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females than in males and decreased significantly with age in both males and females. This analysis also suggested a possible dose-dependent decrease in the number of TRECs in the CD4 T-cell fraction of the survivors who were less than 20 years of age at the time of bombing ($p = 0.09$). A similar statistically significant trend for gender difference or age was observed in the CD8 T-cell fraction of the survivors. However, there was no effect of radiation exposure on the number of TRECs in the CD8-T cell fraction. The results indicate the possibility that A-bomb radiation exposure may have induced a long-term impairment in thymic CD4 T-cell production. Further investigations in a larger study population are necessary to test this hypothesis.

Introduction

Advancing age is accompanied by a variety of alterations in the immune system that can increase the susceptibility of affected individuals to certain diseases. Particularly, age-dependent decreases of T lymphocyte count and function can lead to persistent infections and chronic inflammation. Especially, deficits of the naïve T-cell population may diminish the capability of the host immune system to defend against intrusion of pathogens to which the person has not been previously exposed[11]. Our earlier immunological studies on A-bomb survivors have shown that percentages of naïve CD4 and CD8T cells in peripheral blood lymphocytes are significantly decreased in a radiation dose-dependent manner almost 60 years after the bombing[15]. This may indicate that the naïve T-cell pools poorly recovered after radiation-induced damage of the T-cell system and have never returned to the normal level. Two distinct mechanisms are involved in ensuring immune reconsitution after T-cell depletions, such as those due to radiation or chemotherapy[9]. The first mechanism depends upon renewed proliferation of the survived mature T cells that can repopulate the memory T-cell pool, whereas the second relies upon the differentiation of hematopoietic stem cells into new T cells that comprise the naïve T-cell pool. Therefore we hypothesized that an impairment of the ability to maintain normal-sized naïve-CD4 T-cell pools in A-bomb survivors could have resulted from an insufficient supply of new CD4 T cells from the thymus.

It has become possible to be enumerated the peripheral blood $\alpha\beta$T cells that have recently emigrated from the thymus without having experienced cell divisions in the periphery by quantifying T-cell receptor-rearrangement excision circles (TRECs) generated during T-cell receptor (TCR) gene rearrangement in the thymus[13]. TRECs can be specifically amplified by PCR using primers that anneal to sequences adjacent to the joining signal ends of the DNA excised from the TCR $\alpha\delta$ locus and can be quantified by competitive PCR or real-time PCR[1,2,10]. Because the excised DNA is not replicated and lost in daughter cells, TRECs can be detected primarily in naïve T-cell populations but most merely in memory T cells[1]. Recent studies using quantification of TRECs in humans suggest that thymic function that produces T cells declines with age[1),2)]. Further, in patients infected with HIV-1, the thymic function decreases with progression of the viral burden but can recover to some extent after high active antiretroviral therapy[1]. We therefore assumed that A-bomb radiation may have accelerated age-dependent thymus involution and expected to find that the numbers of T cells with TRECs would be somewhat lower in A-bomb exposed survivors than in age-matched but non-exposed controls. In the present study we are examining the number of TRECs in peripheral blood T-cell populations among A-bomb survivors to test the hypothesis that abnormal decreases in the naïve T-cell populations may have resulted from reduction in thymic T-cell production ability of the survivors, specifically as related to radiation.

Materials and methods

Blood donors

Blood samples were obtained from members of a survivor cohort in which 1,280 survivorshad been selected, as they distributed almost equally by age, gender, and radiation dose, from Hiroshima participants in the Adult Health Study (AHS) at the Radiation Effects Research Foundation (RERF) in 1992[8]. The selected study population consists of Hiroshima survivors who were exposed to radiation doses of ≥ 0.005 Gy (0.005-4 Gy) within two kilometers of the hypocenter and a second group who were > 3 km from the hypocenter (i.e., exposed to background doses). The radiation doses are based on DS02[12]. Each participant is invited to have clinical examination at

RERF every 2 years. For the present study, blood samples were obtained with the informed consent of the survivors and analyzed between May 2003 and May 2005. Besides the survivors, samples from several laboratory controls (five males aged 38-60, and two females aged 39 and 43) were also obtained with the informed consent. Mononuclear cell fractions were separated by the Ficoll-Hypaque gradient technique[7] and used for analyses.

Isolation of CD4 and CD8 T-cell populations

Approximate ten million mononuclear cells were stained with 10μl of FITC-labeled CD4 antibody (BD Biosciences, San Jose, CA, USA) and 25μl PE-Cy5-labeled CD8 (BD-PharMingen, San Diego, CA, USA) in 200μl of PBS containing 1% FCS for 30 min on ice. The cells were washed and resuspended in PBS containing1% FCS and 0.1% sodium azide, and applied to a FACS Vantage SE (BD Biosciences). Each CD4 and CD8 T-cell fraction was separated and collected into a tube containing 100μl of PBS plus 1% BSA (Sigma-Aldrich, St. Louis, MO). In some experiments, approximate ten million mononuclear cells were stained with 10μl of FITC-labeled CD4 antibody and 0.5μl of PE-labeled CD45RA antibody (Coulter-Immunotech, Marseille, France), and $CD4^+$ $CD45RA^+$ naïve and $CD4^+$ $CD45RA^-$ memory T-cell fractions were similarly separated by the FACS Vantage SE. The collected cells were washed with PBS and centrifuged with 3,000 rpm for 5 minutes, the supernatants were removed, and the cell pellets were stored at -20℃ until used for TREC measurement.

Measurement of TREC numbers

The number of TRECs in 1 x 10^5 cells from each fraction were enumerated by the method reported by Yasunaga, et al[16]. The procedure was modified to use crude DNA extracts resulting from a single treatment with proteinase K, which proved to be suited to accurate quantification of the TRECs. In brief, 7μl (7μg) of proteinase K (Sigma-Aldrich) was used per 10^5 cell pellets, and the cells were digested at 56℃ for 2 hr in the presence of 0.02% NP-40, 50mM KCl, 10mM Tris-HCl (pH 8.4) and then incubated at 95℃ for 30 min. Sequences of primers and probes used for DNA amplifications were like those reported by Yasunaga, et al[16]: Primers used for amplification of TREC were 5'-TCCCTTTCAACCATGCTGACA-3' and 5'-TGCCTATGCATCACCGTGC-3'. The probe was 5'-CTCTGGTTTTTGTAAAGGTGCCCACTCCTG-3' labeled with fluorescent FAM at the 5'end and fluorescent TAMRA at the 3' end. To measure cell equivalents in the real-time PCR, recombination activating gene 1 (*RAG-1*) sequence in each sample was quantified by the method similar to that for TREC. The sequences of primers for *RAG-1* exon 2 detection were also similar to those reported by Yasunaga, et al[16]: 5'-CCCACCTTGGGACTCAGTTCT-3' and 5'-CACCCGGAACAGCTTAAATTTC-3', and the probe was 5'-CCCCAGATGAAATTCAGCACCCACATA-3' labeled with FAM (reporter) at the 5' end and TAMRA (quencher) at the 3' end. The crude DNA extracts (7μl) were mixed with 10μl of AbsoluteTM QPCR Mixes (Abgene House, Surrey, UK), and each 1μl of the primer (final concentration, 0.3μM) and probe (final concentration, 0.2μM) was added to the mixture. The PCR conditions were 50 cycles of 15 seconds at 95℃ followed by 60 seconds at 60℃. All experiments were performed and analyzed by the ABI PRISM 7900 Sequence Detection Systems (Applied Biosystems, Foster City, CA). Number of TRECs in each sample was calculated using the following formula.

Number of TREC copies per 10,000 cells

= 10,000 / 2 (cycles required for the significant amplification of TREC) - (cycles required for the significant amplification of *RAG-1*) - 1

Data analysis

A standard multiple-linear-regression method was used to regress log-transformed number of TRECs (per 10^5 cells) on age of the individual at the time of the bombing (ATB), gender and DS02 A-bomb radiation exposure dose. The zero value was impossible of log transformation, and thus the value showing zero for the number of TREC copies per 10,000 cells was replaced with 0.05 when the value was transformed into natural log. All statistical analyses were carried out using the SAS program (SAS Institute Inc., Cary, NC, USA).

Results

Quantification of TRECs by real-time PCR

In our Institute, the amount of blood to collect from A-bomb survivors is limited so as to cause the least amount of anxiety in the donor. We therefore tried to directly

amplify TREC sequences by real-time PCR in crude DNA extracts from lymphocyte fractions that were treated with proteinase K to try to avoid loss of cellular DNA during its purification from a small number of blood cells as few as 10^5. We similarly quantified *RAG-1* copies in each sample by the real-time PCR to measure cell equivalents in the crude extracts. Representative amplification profiles of crude DNA extracts from CD45RA$^+$ naïve and CD45$^-$ memory CD4 T cell fractions isolated from the same individual are shown in Fig. 1. The number of PCR cycles required to give adequate amplification of TREC was much higher in the naïve CD4 T-cell fraction than in the memory CD4 T-cell fraction, whereas the number of cycles to give significant amplification of *RAG-1* was about equal in these T-cell fractions. Similarly, the number of TREC copies per 10,000 cells calculated from the

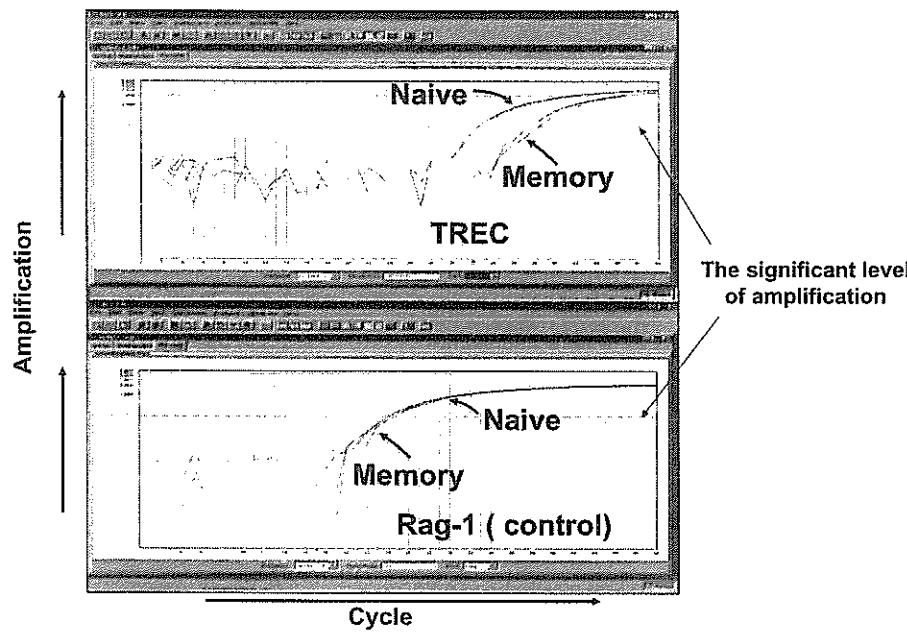

Fig.1 Representative amplification profiles in crude DNA extracts from CD45RA$^+$ naïve and CD45$^-$ memory CD4 T cell fractions isolated from a typical individual. The upper and lower panels show real-time PCR for the TREC and *RAG-1* sequences, respectively.

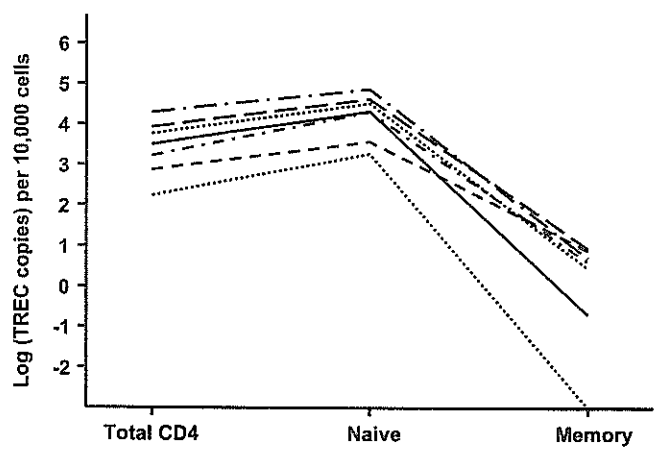

Fig. 2 The number of TREC copies per 10,000 cells in total CD4 T-cell fractions, and naïve and memory CD4 T-cell fractions from seven laboratory controls (five males aged 38-60, and two females aged 39 and 43). Each line indicates the value of the number of TREC copies in each individual.

difference between the cycles for TREC and *RAG-1* amplifications was much higher in the naïve CD4 T-cell fraction than in the memory CD4 T-cell fraction among seven laboratory volunteers (Fig. 2).

To determine whether the real-time PCR methods can quantitatively detect TRECs, we analyzed amplification profiles in samples containing a TREC-negative cell population and graded numbers of $CD45RA^+$ naïve CD4 T cells that were obtained from a young adult and therefore were expected to contain large numbers of TRECs. As shown in Fig. 3, there was a reasonable linear correlation ($r = -0.95$, constant of proportion is -1.02) between the cycles and the number (base 2 logarithm) of naïve CD4 T cells, indicating that the two-fold reduction in the number of naïve CD4 T cells correspond to almost twice increase of the PCR cycle. Furthermore, reasonable reproducibility (coefficient values were 8.2, 18.3, and 21.8 in three individuals) were found in our real-time PCR analyses (Fig. 4). These results provided a basis to believe that the real-time PCR could accurately quantify the numbers of TREC copies in T-cell fractions obtained from A-bomb survivors or any other human population.

TREC analyses among A-bomb survivors

Thus far the numbers of TREC copies in CD4 T-cell fractions from 445 survivors and those in CD8 T-cell fractions from 426 survivors have been examined. Fig. 5

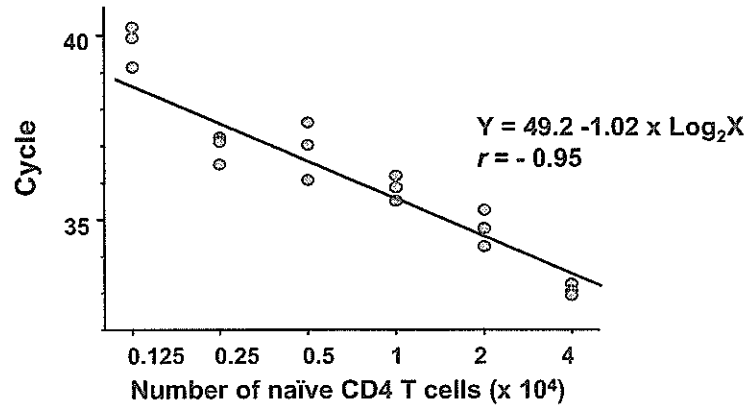

Fig. 3 The relationship between the number of cells containing TRECs and PCR cycles to give the significant amplification of TRECs. Each sample consisted of 10^5 cells containing a TREC-negative cell population and the indicated number of $CD45RA^+$ naïve CD4 T cells that were obtained from a young adult male. The TREC-negative cells were prepared from a T-cell line (KI-19) that was clonally propagated *in vitro* as described [6]. We confirmed that these cells contain no detectable TREC in the real-time PCR.

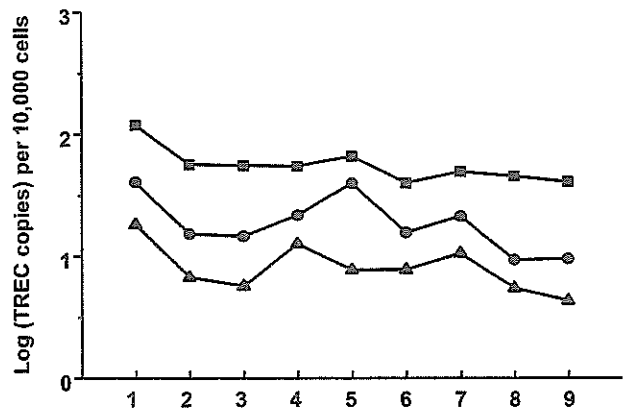

Fig. 4 1Reproducibility in the real-time PCR analyses. Each 10^5 peripheral blood mononuclear cells from three laboratory controls was stored at -20℃ and analyzed nine different times during about one and a half years. Each line indicates the value of each individual.

shows the relationships between the number of TREC copies in the CD4 or CD8 T-cell fraction and the age of the survivors at the time of the bombing (ATB). Although the points are widely scattered, the number of TREC copies significantly ($p < 0.001$) decreased with increase of age ATB in both the CD4 and CD8 T-cell fractions. The same kind of trend was observed when the values were analyzed using age of the survivors at the time of examination instead of age ATB (data not shown). As for the numbers of TREC copies in the CD4 T-cell fraction, however, the individual log-values were normally distributed in survivors who were age ATB < 20 but not when individuals with age ATB ≥ 20 were included. Whereas, no log-normal distribution could be observed as for the numbers of TREC copies in the CD8 T-cell fraction even when those who were age ATB ≥ 20 were excluded. This might be due to the fact that individuals who showed extremely low numbers of TREC copies in the CD4 T-cell fraction were mostly distributed in the age ATB ≥ 20 group while those who showed

Table 1 Multi-regression analysis of effects on the number of TREC copies in CD4 and CD8 T-cell fractions of A-bomb survivors (Age ATB < 20)*

Factors (unit)	% Change	95% CI**	*p*
CD4 (n = 313)			
Dose (1 Gy)	-14.8	-32, 2.4	0.09
Age (10 y)	-50.8	-27.1, -74.5	< 0.001
Gender (M:1, F:2)	77.2	48.3, 106	< 0.001
CD8 (n = 300)			
Dose (1 Gy)	-19.2	-49.8, 11.4	0.2
Age (10 y)	-74.4	-31.8, -117	< 0.001
Gender (M:1, F:2)	287	235, 339	< 0.001

*Associations of the number of TREC copies with age at the time of bombing (age ATB), gender, and radiation dose (dose) were analyzed based on a multiple regression model.

**CI = confidence interval

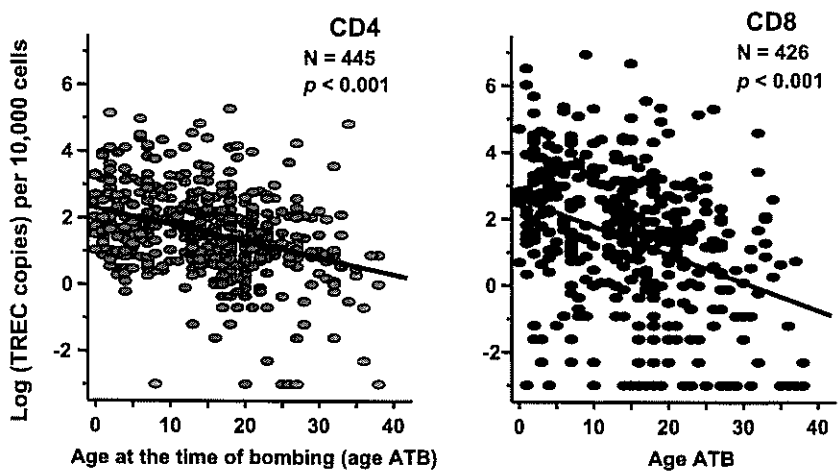

Fig. 5 The number of TREC copies in the CD4 (left panel) or CD8 (right panel) T-cell fraction in each individual is plotted against the age of individual at the time of the bombing (ATB). Each line denotes the regression between the number of TREC copies and age ATB.

extremely low numbers of TREC copies in the CD8 T-cell fraction appeared in both younger and older age ATB groups (Fig. 5). There was a strong correlation ($r = 0.7$) between the numbers of TREC copies in the CD4 and CD8 T-cell fractions from the same survivors who were age ATB < 20, indicating that individual ability of thymic CD4 T-cell production may be somewhat proportional to that of thymic CD8 T-cell production at least in the age ATB < 20 group.

A multiple regression analysis was conducted for the number of TREC copies in the CD4 or CD8 T-cell fraction among survivors who were age less than 20 ATB, because the individual TREC number in this group appeared to be close to the normal distribution (especially in the CD4 T-cell fraction) and to be strongly affected by age ATB or gender. The results are summarized in Table 1. The number of TREC copies in the CD4 T-cell fraction decreased significantly with age ATB ($p < 0.001$) and was higher in females than in males ($p < 0.001$). These data also suggested that there might be a dose-dependent decrease in the number of TRECs in the CD4 T-cell fraction of the survivors ($p = 0.09$). Similar statistically significant trend for age ATB ($p < 0.001$) and gender difference ($p < 0.001$) were observed in the CD8 T-cell fraction of the survivors. The number of TRECs in the CD8 T-cell fraction of the survivors appeared to decrease somewhat with radiation dose, but this dose trend was not statistically significant ($p = 0.2$).

Discussion

Our present study suggests that the numbers of TREC copies in both CD4 and CD8 T-cell fractions decrease with age. TREC copy number is higher in females than in males. We believe this to be the first report of gender difference in TREC copy number in a human population. Because our study population consisted of persons aged over 58 years, it is likely that age-dependent decline in human thymic output represents a characteristic of aging among the elderly. Because thymic involution resulting in reduction of cellularity in this organ is known to be a primary cause of age-dependent decline in TREC copy numbers in both CD4 and CD8 T-cell populations[17], the present results suggest that thymus involution may continue even after age 58. The observed gender difference as these populations age suggests that the aging process differently affects thymic involution in males and females. A further analysis including younger subjects is needed to test this hypothesis.

Our previous studies[7, 8, 15] indicated that the size of naïve cell pools in CD4 T-cell populations was reduced possibly as a consequence of radiation exposure among A-bomb survivors. One of the most plausible mechanisms for size reduction of naïve T-cell populations could have resulted from insufficient supply of new T cells from the thymus, since the majority of naïve T cells develop in the thymus. We therefore expected to find similar reduction in number of recent thymic emigrant cells in our latest study population. Even though the current study is preliminary and radiation effect was suggested only among survivors who were age < 20 years ATB, the result for number of TREC copies in CD4 T-cell populations supports the possibility that A-bomb radiation exposure can induce long-term impairment of CD4 T-cell production. Actually, number of TREC copies appeared to positively correlate ($p < 0.001$, $r = 0.3$) with proportion of $CD45RA^+$naïve cells in the CD4 T-cell population among the 443 survivors available for examination for both TREC number and CD45RA expression in their CD4 T-cell populations (data not shown). Therefore, it is reasonable to assume that radiation-induced reduction in production of new CD4 T cells in the thymus could cause impaired maintenance of naïve CD4 T-cell pools among radiation-exposed individuals.

It has been reported[14] that significantly fewer CD4 T cells containing TRECs are seen in bone marrow transplantation patients exposed to whole-body irradiation more than 20 years ago, even though the doses (10 Gy or more) far exceeded those received by A-bomb survivors. Because reconstituted hematopoietic cell populations of such patients are almost entirely derived from unirradiated donors, and with their own thymus epithelial cells severely irradiated, reduction in number of TREC copies may be due to radiation effect on the ability of thymus epithelial cells to support thymopoiesis. In an earlier study[5] using mouse models, it was shown that there are radiation-dose-dependent decreases in T-cell regeneration activity in mice subjected to thymectomy followed

by transplantation with irradiated thymuses and in those that underwent local thymic irradiation. Effects of irradiation on mice undergoing bone marrow transplantation[3] are also noted for dose-dependent reductions in the production of IL-7, which is essential for T-cell development, and in the number of MHC class II^+ epithelial cells, which are the primary source of IL-7. It is therefore hypothesized that A-bomb radiation damaged thymic stroma, in which epithelial cells primarily support thymopoiesis, and induced long-term impairment in the ability of the thymus to supply new T cells containing TRECs into the periphery.

Another hypothesis to be tested is that reduction of TREC copy number in the CD4 T-cell populations of A-bomb survivors might have resulted from dilution of TREC-bearing cell populations by homeostatic and/or antigenic proliferations of naïve T-cell populations followed by their transfer to memory T-cell pools. It is known that such proliferations of naïve T cells are largely dependent on their interaction with dendritic cells[4]. We are planning to evaluate the *in vivo* cell division frequencies of both naïve and memory CD4 T-cell populations by analyzing telomere lengths in these T-cell populations among A-bomb survivors. We will also explore a potentially valuable method for analyzing dendritic-cell reconstitution following T-cell depletion by radiation.

Despite that similar mechanisms are plausible for the size reduction of naïve CD8 T -cell populations that we reported among A-bomb survivors[15], we did not observe in the present study any statistically significant or even suggestive association between numbers of TREC copies in CD8 T-cell populations and radiation dose. This could reflect that the effect of aging and/or gender on TREC counts is more pronounced in CD8 T-cell populations than in CD4 T-cell populations (Table 1), which would obscure the effect of radiation *per se* on CD8 T-cell populations among elderly survivors. We also noted that the proportion of naïve CD8 T cells in peripheral blood lymphocyte populations was as low as a few percent, whereas that of naïve CD4 T cells ordinarily exceeds 10 percent among our study's elderly survivor population[15]. Thus, it is expected that naïve CD8 T cells containing TREC in T-cell fractions we analyzed are very infrequent when compared with naïve CD4 T cells containing TREC. More sophisticated statistical analyses with additional samples will be necessary to evaluate possible radiation effects on such a small number of cells per sample. Such an approach would also provide improved evidence regarding possible long-term effects of radiation exposure on the human T-cell system involving both CD4 and CD8 T-cell populations.

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Role of Single-stranded DNA in Targeting REV1 to Primer Termini*[S]

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Cellular functions of the *REV1* gene have been conserved in evolution and appear important for maintaining genetic integrity through translesion DNA synthesis. This study documents a novel biochemical activity of human REV1 protein, due to higher affinity for single-stranded DNA (ssDNA) than the primer terminus. Preferential binding to long ssDNA regions of the template strand means that REV1 is targeted specifically to the included primer termini, a property not shared by other DNA polymerases, including human DNA polymerases α, β, and η. Furthermore, a mutant REV1 lacking N- and C-terminal domains, but catalytically active, lost this function, indicating that control is not due to the catalytic core. The novel activity of REV1 protein might imply a role for ssDNA in the regulation of translesion DNA synthesis.

The majority of both spontaneous and DNA damage-induced mutations in eukaryotes results from replication processes in which REV1, REV3, and REV7 proteins play major roles. Studies of *REV* genes originated with the isolation of yeast *rev* mutants (1, 2), which exhibited a reduced frequency of mutations following treatment with a variety of DNA-damaging agents (3). Deoxycytidyltransferase activity of the REV1 protein and DNA polymerase activity of the REV3-REV7 complex for translesion DNA synthesis were discovered in the pioneering work of Lawrence and co-workers (4, 5). By using information obtained from yeast studies, homologues of the encoding genes were subsequently identified in mammals (6–13), and it is now well established that the pathway has been conserved in evolution from the yeast to humans.

REV1 is a member of the Y family of DNA polymerases, which also includes DNA polymerase (pol)[2] IV and V in *Escherichia coli*, and DNA pol η, ι, and κ in eukaryotes (14, 15). These proteins are required for translesion DNA synthesis because many lesions block typical replicative DNA polymerases. However, the REV1 protein almost exclusively utilizes only dCTP, in contrast to the other members of the family, and preferentially inserts dCMP opposite template G and a variety of damaged bases and apurinic/apyrimidinic sites (4, 6, 7, 11, 16–18). Because of this preference, REV1 has been called a deoxycytidyltransferase (3, 4). This novel activity has been maintained throughout eukaryotic evolution, implying a contribution to survival (3). Recently, it was demonstrated that the specificity for dCMP is tightly regulated by formation of hydrogen bonds with an arginine residue in the protein, but not template G (19). Indeed, dCMP residues are known to be incorporated opposite apurinic/apyrimidinic sites in the majority of bypass events in wild type yeast cells but not the rev1Δ strain (20–23).

A second function of the *REV1* gene product in the mutagenesis pathway has also been proposed, independent of its action as a deoxycytidyltransferase (24). Methyl methanesulfonate-induced mutagenesis has been shown to be normal in a site-directed mutant lacking deoxycytidyltransferase activity (25). Furthermore, although the REV1 protein does not allow bypass of thymine-thymine (6-4) photoproducts *in vitro*, the gene is required for bypass replication of this lesion in yeast cells (24, 26, 27). With respect to the second function, the BRCA1 C-terminal domain of REV1 has an essential function, mutation abolishing UV-induced mutagenesis, even if the protein retains normal levels of transferase activity *in vitro* (24, 27).

Evolutionary preservation of functions of the mammalian *REV1* gene was first indicated by the finding that human cell lines expressing high levels of human *REV1* antisense RNA exhibit a much reduced frequency of 6-thioguanine-resistant mutants induced by UV light (10). This feature was confirmed in another experimental system using a ribozyme that cleaves human *REV1* mRNA (28) and with an RNA interference down-regulating mouse Rev1 function (29). Furthermore, mouse embryonic stem cells carrying a mutation lacking the BRCA1 C-terminal domain of the *Rev1* gene also exhibit sensitivity to a wide range of DNA-damaging agents and a reduced level of UV light-induced mutations (30, 31). Recently, chicken Δ*Rev1*-DT40 cell lines were generated and found to exhibit slow growth and sensitivity to a wide range of DNA-damaging agents (32–34).

Although studies of the cellular functions of the *REV1* gene in yeast to humans have shed light on its importance for maintaining genetic integrity, the biochemical basis is poorly understood. It is known that both mouse and human REV1 proteins interact with REV7 and other Y family polymerases through the same C-terminal region (35–38). In particular, the REV1-REV7 interaction is very stable and results in formation of a heterodimer in yeast and humans (39, 40). The C-terminal region

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[2] The abbreviations used are: pol, DNA polymerase; ssDNA, single-stranded DNA; BSA, bovine serum albumin.

of yeast Rev1 is also required for stimulation of yeast pol ζ (41), although the amino acid sequence is not conserved. From these observations, a noncatalytic role in translesion DNA synthesis has been proposed (35–41).

In this report, we document a novel biochemical property of human REV1. We show that REV1 has single-stranded DNA (ssDNA) binding activity with affinity much higher than that of the primer-template. After binding to ssDNA, REV1 can translocate and be targeted to primer termini. We consider that this property might reflect a particular biochemical function required for regulation of the repair pathway involved in lesion bypass replication.

EXPERIMENTAL PROCEDURES

Oligonucleotides—The oligonucleotide sequences were as follows: H1, 5′-GACGCTGCCGAATTCTGGCTTGCTAGGACATCTTTGCCCACGTTGACCCG-3′; H2, 5′-CGGGTCAACGTGGGCAAAGATGTCCTAGCAATGTAATCGTCTATGACGTC-3′; H3, 5′-GACGTCATAGACGATTACATTGCTAGGACATGCTGTCTAGAGACTATCGC-3′; and H4, 5′-GCGATAGTCTCTAGACAGCATGTCCTAGCAAGCCAGAATTCGGCAGCGTC-3′. The primer-template, P5786T, was made by annealing the 5′-^{32}P-labeled primer P5786 (5′-GTCTACAAGTTCAC-3′) with the template 5786T (5′-ATTCTGAGCAGCCCGGATGGTGAACTTGTAGAC-3′). Others are shown in Fig. 3*A*.

Plasmids—Human *POLB* cDNA was amplified from HeLa cDNA by PCR and inserted into the NdeI-BamHI site of a pET20b(+) vector (Novagen) to yield plasmid pET-POLB. Human *POLH* cDNA was amplified by PCR from a plasmid carrying human *POLH* cDNA, kindly provided by Dr. F. Hanaoka, Osaka University, Osaka, Japan (42), and inserted into the NdeI-BamHI site of a pET15b vector (Novagen) to yield pET-h6-POLH. Human *POLA* cDNA was amplified by PCR from a plasmid carrying human *POLA* cDNA, kindly provided by Dr. M. Suzuki, Nagoya University, Nagoya, Japan (43), and inserted into the NdeI-XhoI site of a pET15b vector (Novagen) to yield pET-h6-POLA. Human *POLA2* cDNA was amplified from HeLa cDNA by PCR and inserted into the NdeI-KpnI site of a pCDFK vector, which was made by deletion of an EcoNI-AflII fragment and replacement of the streptomycin resistance gene of pCDFDuet™-1 (Novagen) with the kanamycin resistance gene from pSY343 (44), to yield pCDFK-POLA2. The nucleotide sequences were verified in all these plasmids.

Proteins—Intact REV1 was purified as described (17), along with mutants (6). Human pol β was overproduced in BL21 (DE3) (45) harboring pET-POLB and similarly purified (46).

His-tagged human pol η (h6-pol η) was purified from overexpressing *E. coli* cells as follows. BL21 (DE3) (45) harboring pET-h6-POLH was grown in 500 ml of LB medium supplemented with ampicillin (250 mg/ml) at 15 °C with aeration until the culture reached an A_{600} value of 0.6. Isopropyl β-D-thiogalactopyranoside was added to 0.2 mM, and incubation was continued for 10 h. The resultant cell paste was resuspended in 2 ml of buffer I (50 mM HEPES-NaOH, pH 7.5, 0.1 mM EDTA, 10 mM β-mercaptoethanol) containing 1 M NaCl per 1 g of cells and frozen in liquid nitrogen. The cells were thawed in ice water and lysed after addition of phenylmethylsulfonyl fluoride to 0.1 mM by introduction of 100 mM spermidine and 4 mg/ml lysozyme in buffer I containing 1 M NaCl, to 10 mM and 0.4 mg/ml, respectively. The cells were incubated on ice for 30 min, heated in a 37 °C water bath for 2 min, and further incubated on ice for 30 min at 4 °C. Then the lysate was clarified by centrifugation at 85,000 × *g* for 30 min at 4 °C. Subsequent column chromatography was carried out at 4 °C using a fast protein liquid chromatography system (GE Healthcare). After adding imidazole to 50 mM, the lysate was applied at 0.1 ml/min to a 1-ml HiTrap chelating column (GE Healthcare), which had been treated with 0.1 M $NiSO_4$ and then equilibrated with buffer A (50 mM HEPES-NaOH, pH 7.5, 10 mM β-mercaptoethanol, 10% glycerol) containing 1 M NaCl and 50 mM imidazole. The column was washed with 10 ml of equilibration buffer, and then 10 ml of buffer A containing 1 M NaCl and 100 mM imidazole, and the h6-pol η was eluted with 10 ml of a linear gradient of 100–300 mM imidazole in buffer A containing 1 M NaCl. Fractions containing the enzyme were pooled and concentrated and then loaded at 0.1 ml/min onto a Superdex 200 HR 10/30 column (GE Healthcare) equilibrated with buffer A containing 1 M NaCl. The h6-pol η peak fractions were pooled, frozen in liquid nitrogen, and stored at −80 °C.

His-tagged human pol α p180 (h6-p180) was purified as a complex with p70 from overexpressing *E. coli* cells. BL21 (DE3) (45) harboring both pET-h6-POLA and pCDFK-POLA2 was grown in 10 liters of "terrific" broth (47) supplemented with ampicillin (250 mg/ml) at 15 °C with aeration until the culture reached an A_{600} value of 0.6. Isopropyl β-D-thiogalactopyranoside was added to 0.2 mM, and the incubation was continued for 5 h. The resultant cell paste was resuspended in 2 ml of buffer I containing 0.5 M NaCl per 1 g of cells and frozen in liquid nitrogen. The cells were thawed in ice water and lysed after addition of phenylmethylsulfonyl fluoride to 0.1 mM by introduction of 100 mM spermidine and 4 mg/ml lysozyme in buffer I containing 0.5 M NaCl, to 10 mM and 0.4 mg/ml, respectively. The cells were incubated on ice for 30 min, heated in a 37 °C water bath for 2 min, and further incubated on ice for 30 min at 4 °C. Then the lysate was clarified by centrifugation at 85,000 × *g* for 30 min at 4 °C. Subsequent column chromatography was carried out at 4 °C using a fast protein liquid chromatography system. After adding imidazole to 50 mM, the lysate was applied at 0.5 ml/min to a 1-ml HiTrap chelating column, which had been treated with 0.1 M $NiSO_4$ and then equilibrated with buffer A containing 0.5 M NaCl and 50 mM imidazole. The column was washed with 10 ml of equilibration buffer at 0.1 ml/min and then eluted with 10 ml of a linear gradient of 50–100 mM imidazole in buffer A containing 0.5 M NaCl. Fractions containing h6-p180-p70 were pooled, diluted with buffer A to 100 mM of NaCl, and applied at 0.1 ml/min to a 1-ml HiTrap Q HP column (GE Healthcare) equilibrated with buffer A containing 100 mM NaCl. The column was washed with 10 ml of equilibration buffer, and the h6-p180-p70 complex was eluted with 10 ml of a linear gradient of 100–500 mM NaCl in buffer A. Fractions containing h6-p180-p70 were pooled and loaded at 0.1 ml/min onto a Superose 6 HR 10/30 column (GE Healthcare) equilibrated with buffer A containing 0.5 M NaCl. The h6-p180-p70 peak fractions were pooled, frozen in liquid nitrogen, and

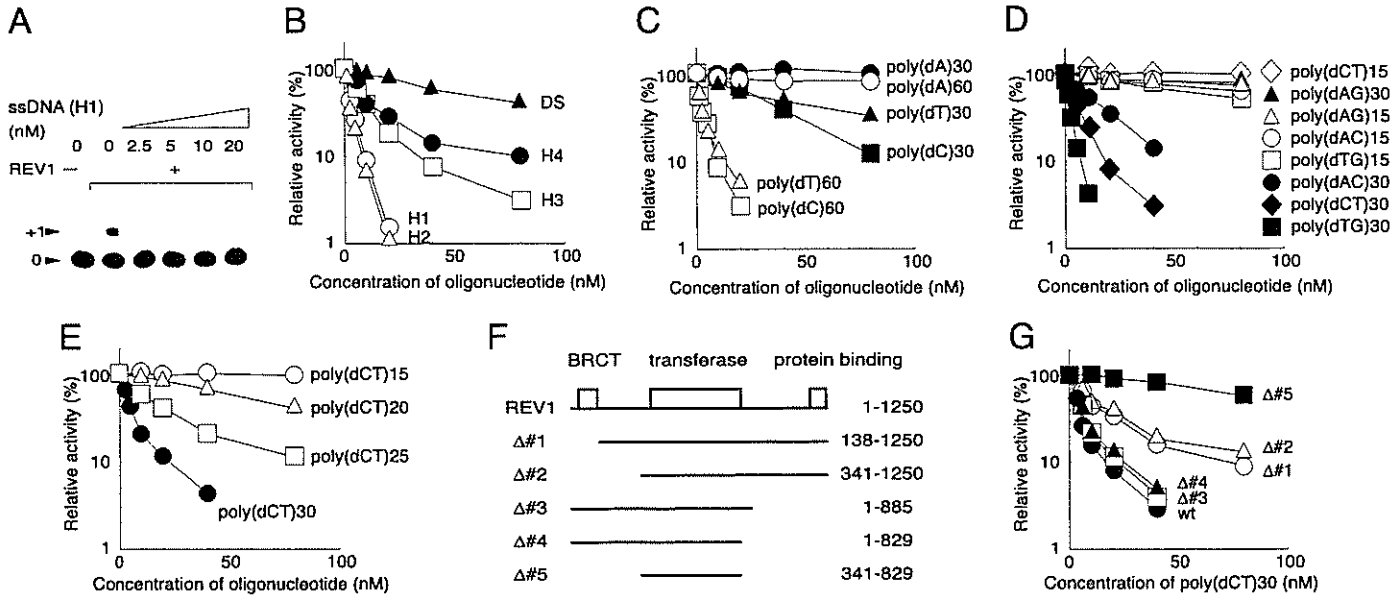

FIGURE 1. **Inhibition of dCMP transferase activity of human REV1 protein by ssDNA.** Inhibition of dCMP transferase activity of the REV1 protein (*A–E*) or its deletion derivatives (*G*) by various oligonucleotides is shown. Ten ng of REV1 or deletion derivatives (except for 5 ng of Δ5) and the primer-template, P5786T (100 nM), were incubated under standard reaction conditions in the presence of the indicated concentration of oligonucleotides. The double-stranded DNA represented as *DS* in *B* was made by annealing H3 and the complementary oligonucleotide. The reaction products were resolved on 20% polyacrylamide gels containing 8 M urea and autoradiographed at −80 °C (*A*, *0* and *+1* represent positions of substrate and product, respectively), and the amounts of DNA present in each band were quantified (*B–E* and *G*). *F*, schematic representation of deletion mutants. The molar concentration of REV1 in the reactions was 2.8 nM and those of mutant REV1 were 3.1 nM Δ1, 3.8 nM Δ2, 3.9 nM Δ3, 4.2 nM Δ4, and 3.6 nM Δ5.

stored at −80 °C. Protein concentrations were determined by protein assay using BSA (Bio-Rad) as the standard.

Primer Extension Assay—The primers were labeled using polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (GE Healthcare) and annealed to the respective templates. The standard reaction mixture (25 μl) contained 50 mM Tris-HCl buffer, pH 8.0, 2 mM $MgCl_2$, 0.1 mg/ml BSA, 5 mM dithiothreitol, 0.1 mM dCTP, 100 nM primer-template, and 1 μl of protein sample diluted with buffer (50 mM HEPES-NaOH, pH 7.5, 500 mM NaCl, 10 mM β-mercaptoethanol, 10% glycerol, 0.1 mg/ml BSA) as indicated. After incubation at 30 °C for 10 min, reactions were terminated with 10 μl of stop solution (30 mM EDTA, 94% formamide, 0.05% bromphenol blue, 0.05% xylene cyanol), and products were resolved on 20% polyacrylamide gels containing 8 M urea and autoradiographed at −80 °C. The amount of DNA present in each band was quantified using a Bio-Imaging Analyzer BAS2000 (Fuji Photo Film Co., Ltd.). The conditions for the primer extension assay for DNA polymerase shown in Fig. 4*B* were identical to those for the dCMP transferase assay.

DNA Polymerase Assay—DNA polymerase activities shown in Fig. 4*A* were measured by incorporation of [α-^{32}P]dCMP using the unlabeled primer-template, P5786T, as a substrate. The reaction mixture (25 μl) contained 50 mM Tris-HCl buffer, pH 8.0, 2 mM $MgCl_2$, 0.1 mg/ml BSA, 5 mM dithiothreitol, 0.1 mM each of dGTP, dATP, dTTP, and [α-^{32}P]dCTP (GE Healthcare), 100 nM primer-template (P5786T), and 1 μl of protein sample diluted with buffer (50 mM HEPES-NaOH, pH 7.5, 500 mM NaCl, 10 mM β-mercaptoethanol, 10% glycerol, 0.1 mg/ml BSA) as indicated. Ten ng of REV1, 3 ng of pol α, 2 ng of pol β, and 10 ng of pol η were used in 25-μl reaction mixtures. After incubation at 30 °C for 10 min, reactions were terminated with 10 μl of 30 mM EDTA, and then 1-μl samples were spotted on DE81 paper (Whatman), which was washed three times with 0.5 M Na_2HPO_4. The amount of incorporated [α-^{32}P]dCMP was determined as the radioactivity retained on the paper (48) and quantified using a Bio-Imaging Analyzer BAS2000 (Fuji Photo Film Co., Ltd.)

Electrophoretic Mobility Shift Assay—Poly[d(C-T)] oligonucleotides with various lengths were labeled using polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (GE Healthcare). Assays of DNA binding were performed with modification of a method described previously (6). Reaction mixtures (10 μl) contained 50 mM Tris-HCl buffer, pH 8.0, 2 mM $MgCl_2$, 0.2 mg/ml BSA, 5 mM dithiothreitol, 0.1 mM dCTP, 50 pM oligonucleotide, and 1 μl of protein sample diluted with buffer (50 mM HEPES-NaOH, pH 7.5, 500 mM NaCl, 10 mM β-mercaptoethanol, 10% glycerol, 0.1 mg/ml BSA) as indicated. Incubation was on ice for 20 min followed by loading on prerunning 4% polyacrylamide gels (79:1 acrylamide/bisacrylamide). The electrophoresis buffer contained 6 mM Tris-HCl, pH 7.5, 5 mM sodium acetate, and 0.1 mM EDTA, and the gels were subjected to a constant voltage of 8 V/cm for 2 h at 6 °C. Following gel electrophoresis, the gels were dried and autoradiographed at −80 °C.

RESULTS

High Affinity Binding of REV1 to ssDNA—During biochemical characterization of the dCMP transferase reactions of human REV1 protein, we found a synthetic oligonucleotide, H1, to be a strong inhibitor of the transferase activity of the REV1 (Fig. 1*A*). In reactions containing 100 nM primer-template and 2.8 nM REV1, the transferase activity dropped to less than 10% in the presence of 10 nM of the H1 (Fig. 1, *A* and *B*). To

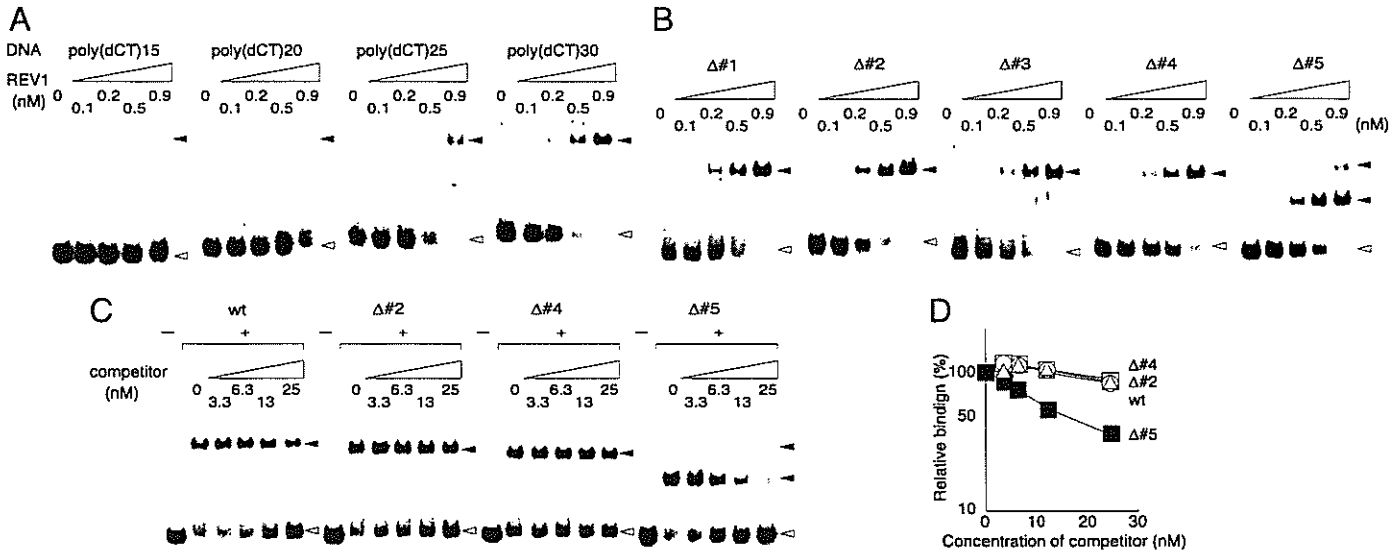

FIGURE 2. **Analysis by electrophoretic mobility shift assay of ssDNA binding to the REV1 protein.** *A* and *B*, electrophoretic mobility shift assays of ssDNA binding to REV1 (*A*) and its deletion derivatives (*B*). Poly[d(C-T)] consisting of the indicated repeats (*A*) or $d(C\text{-}T)_{30}$ 60-mer (*B*) were incubated with REV1 or deletion mutants at the indicated concentrations. *Filled* and *open arrowheads* indicate the positions of the DNA-REV1 complex and the free DNA, respectively. *C*, competition assay of REV1-ssDNA binding activity. In binding reactions with 0.9 nM of REV1 or deletion derivatives, the indicated concentrations of unlabeled primer-template, P5786T, were incubated as a competitor. *D*, quantified results of *C*.

ascertain whether the inhibitory effect was because of a specific nucleotide sequence, oligonucleotides of different sequences (H2–H4) were tested (Fig. 1*B*). The results suggested that the inhibitory effect was not because of a specific nucleotide sequence, although each oligonucleotide exhibited a different extent of inhibition. When the oligonucleotide was annealed with the complementary oligonucleotide and converted to double-stranded DNA, such an effect was much decreased (Fig. 1*B*). The remaining effect might be due to trace contamination of ssDNA (data not shown). To exclude the possibility that the effect is due to local secondary structures formed by the oligonucleotides, we tested 30- and 60-mer oligonucleotides composed of one or two nucleotides, which are guaranteed not to form secondary structures (Fig. 1, *C* and *D*). The results revealed general features for the inhibition. First, it was not because of secondary structures of the oligonucleotides. Second, the composition of the nucleotides affected the extent of inhibition, whereas polypurines, poly(dA) and poly[d(A-G)], showed no effect. Third, the extent of inhibition was stronger with 60- than 30-mer oligonucleotides with the same composition of nucleotides. Furthermore, we systematically addressed the effect of the length using poly[d(C-T)] as a model oligonucleotide (Fig. 1*E*) and found the extent of inhibition to correlate synergistically with the length. In a control experiment, we demonstrated that REV1 could not transfer dCMP to the 3′ end of the $d(C\text{-}T)_{30}$, 60-mer oligonucleotide, under those reaction conditions (supplemental Fig. 1), indicating that the inhibition is not because of random priming reactions with the oligonucleotide. In following experiments, we used poly[d(C-T)] oligonucleotides as model ssDNA substrates.

The inhibitory effect might be due to high affinity binding of the REV1 to the ssDNA, and consequently, it should compete with the primer-template. To examine REV1 ssDNA binding activity, we performed gel mobility shift assays using poly[d(C-T)] oligonucleotides with various lengths as substrates and detected REV1-ssDNA complexes (Fig. 2*A*). When we tested the $d(C\text{-}T)_{15}$, 30-mer oligonucleotide, as a substrate, no complexes could be detected. However, when the length was increased, complexes became visible and were stable. The apparent affinity of REV1 for the oligonucleotides proved to be relative to their lengths. On the 60-mer oligonucleotide, the apparent affinity was very high with less than 1 nM of estimated K_d. Most importantly, the degree of apparent affinity of REV1 for the oligonucleotides of various lengths showed good agreement with the degrees of their inhibitory effects on transferase activity (Fig. 1*E*).

Analysis of Truncated REV1 Proteins—Next, we examined the properties of truncated REV1 proteins (Δ1–Δ5) (Fig. 1*F*) with intact transferase activity (Fig. 3*E*, *panel a*). First, the inhibitory effects of 60-mer poly[d(C-T)] were examined (Fig. 1*G*). The effect of truncation of the C-terminal Δ3 and Δ4 was the same as observed with the full-length protein. On the other hand, truncation of the N-terminal Δ1 and Δ2 resulted in partial resistance to ssDNA. However, truncation of both N- and C-terminal Δ5 showed a much greater effect. The activity of the mutant protein Δ5 consisting of only the transferase domain was not inhibited by the oligonucleotide, indicating inhibition by ssDNA to be modulated by domains outside the transferase domain of REV1.

Then we tested ssDNA binding activity of the mutants (Fig. 2*B*). Surprisingly, binding of the 60-mer poly[d(C-T)] to all the truncated proteins was essentially identical to that to full-length REV1 (Fig. 2*B*). With the full-length REV1, the transferase activity could be inhibited by ssDNA binding. However, this was not the case with truncated protein Δ5. Therefore, we addressed the question of whether the primer-template is accessible to REV1-ssDNA complexes (Fig. 2, *C* and *D*). If REV1 could interact with a primer-template after forming a complex with ssDNA, the complex would be sensitive to an addition of a large amount of the primer-template. In the REV1-ssDNA

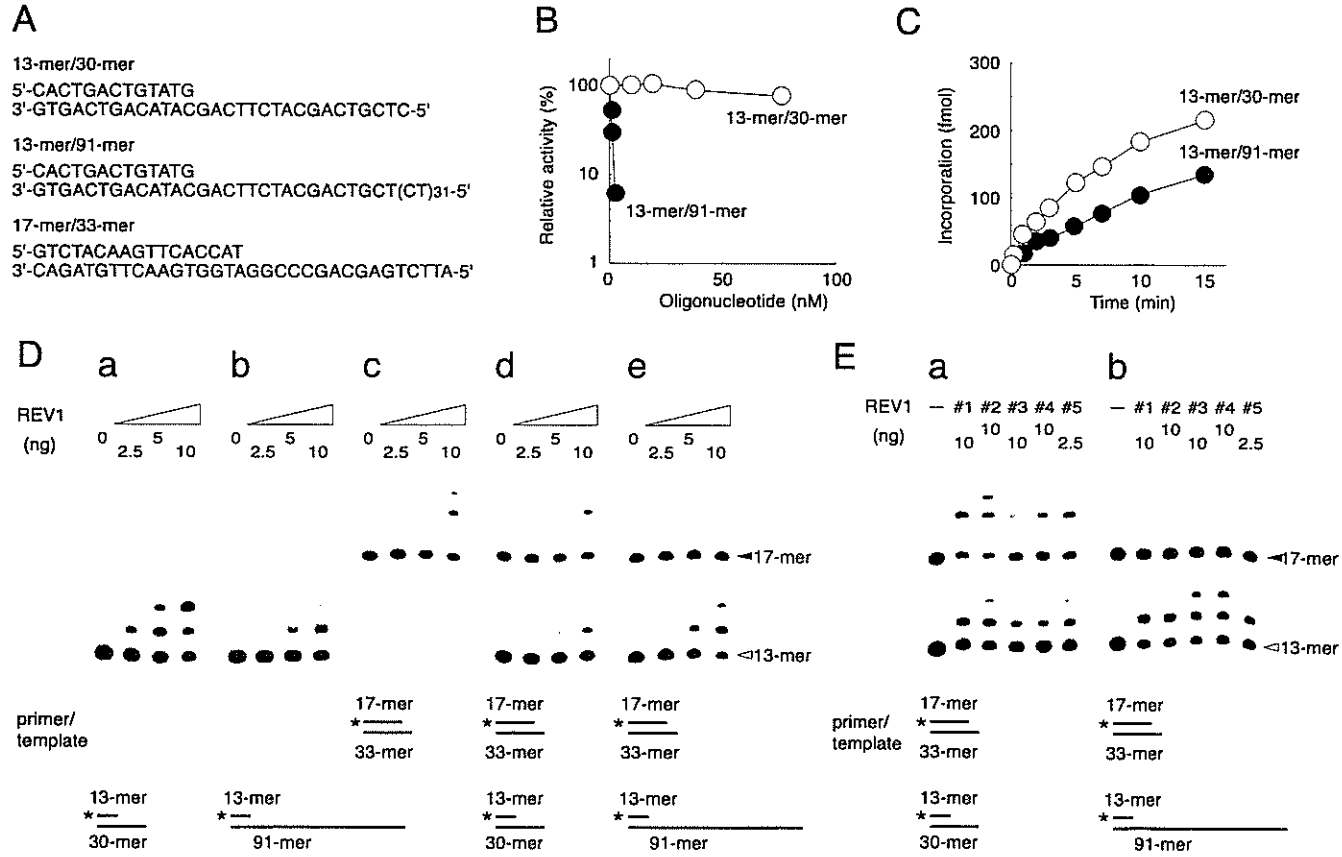

FIGURE 3. **Template specificity of dCMP transferase reactions of REV1.** *A*, nucleotide sequences of the primer-templates. *B*, inhibition of dCMP transferase activity of the REV1 protein by a primer-template containing long ssDNA. Ten ng of REV1 (2.8 nM) and the primer-template, P5786T (100 nM) as a substrate, were incubated under standard reaction conditions in the presence of the indicated concentration of 13/30- or 13/91-mer (*A*). Incorporation into the P5786T was measured and plotted in the graph. *C*, time course of dCMP transferase reactions using 13/30- and 13/91-mer (*A*) as substrates. REV1 (0.7 nM) and each primer-template (20 nM) were incubated for the indicated times. Incorporation into each 13-mer primer was measured. The errors in this assay were less than 5%. In the assays of *B* and *C*, the reaction products were resolved on 20% polyacrylamide gels containing 8 M urea, and amounts of DNA present in each band were quantified. *D* and *E*, competition assay of dCMP transferase activity using various primer-templates. The primer-templates shown in *A* (20 nM each) were used as substrates for the transferase assays (shown at the bottom of each panel) and were incubated with the indicated amount of REV1 (*D*) or deletion derivatives (*E*). The positions of 13- and 17-mer primer on the gels are shown by *open* and *closed arrowheads*, respectively. In the reactions with mixtures of two primer-templates, the respective primers were distinguished by differing size (*panels d* and *e* of *D*, and *panels a* and *b* of *E*). The reaction products migrating between 13- and 17-mer were derived from the 13-mer primer and those migrating larger than 17-mer were derived from the 17-mer primer. The molar concentrations of REV1 in the reactions were 0.7 nM (2.5 ng), 1.4 nM (5 ng), and 2.8 nM (10 ng), and those of mutant REV1 were 3.1 nM Δ1 (10 ng), 3.8 nM Δ2 (10 ng), 3.9 nM Δ3 (10 ng), 4.2 nM Δ4 (10 ng), and 1.8 nM Δ5 (2.5 ng).

binding reaction, a primer-template was therefore introduced as a competitor. The result clearly demonstrated that the Δ5-ssDNA complex was sensitive to addition of primer-template. The amount of the complex was decreased to 30% by addition of primer-template at 25 nM, indicating that the primer-template could access the catalytic site of Δ5 even after complex formation with ssDNA. In contrast, we could not detect any difference between full-length REV1 and Δ2, even though Δ2 exhibited decreased sensitivity to ssDNA (Fig. 1*G*) as compared with full-length REV1. We consider that the difference could be due to sensitivity of the assay systems to detect competition between primer-template and ssDNA, but the results from both experiments were essentially consistent. From the results, we conclude that ssDNA binding itself does not fill up the catalytic site of the transferase but rather prevents accession of primer-template, and this function is because of the presence of N- and C-terminal domains.

Specific Utilization of a Primer-Template Containing Long ssDNA—For further investigation of this novel property of the REV1 protein, we addressed whether the catalytic site of the transferase reaction might be accessible to a primer terminus annealed with a template containing a long ssDNA region (*cis* effect of ssDNA). Because the apparent binding affinity of REV1 to ssDNA is much higher than to the primer terminus (6), if the template contained a long ssDNA region, it could be a target for REV1 binding and inhibit the transferase reaction. To test this possibility, we made two primer-templates: one a 30-mer template annealed with a 13-mer primer, and the other a 91-mer template in which a dCT repeat was attached to the 5′ end of the 30-mer, and the same 13-mer was annealed (Fig. 3*A*). First, we examined whether the primer-template could inhibit REV1 in an ssDNA-dependent manner when it was introduced in the reaction in *trans* (Fig. 3*B*). The transferase activity of REV1 was monitored using a different primer-template, P5786T. The result demonstrated that the oligonucleotide, 13/91-mer, inhibited transferase activity when introduced in *trans* (Fig. 3*B*), the effect being much stronger than that of poly[d(C-T)] 60-mer (Fig. 1*E*). This agreed with the synergistic properties (Fig. 1*E*), considering that the single-stranded region of 13/91-mer is 78 bases. This inhibition was not because of the structure