to atherosclerosis. In addition, chronic kidney disease–related metabolic disorders may affect normal or mildly injured vessels to a lesser extent in normotensives than in hypertensives, and therefore, the 12-year follow-up period of our study may be insufficient to allow for the occurrence of cardiovascular disease.

In the present study, chronic kidney disease was found to be an independent risk factor in men for the occurrence of coronary heart disease but not of ischemic stroke. A possible reason for this discrepancy is competition among causes of cardiovascular disease, whereby our men with chronic kidney disease were more likely to suffer from coronary heart disease than from ischemic stroke, thereby causing possible censorship of data due to coronary death. Also, risk factors may have been modified in response to medical advice and treatment after coronary heart disease events, which would probably have weakened the association between chronic kidney disease and ischemic stroke. In contrast, for women with chronic kidney disease, an opposite phenomenon was observed: the risk of ischemic stroke was significantly elevated, while the risk of coronary heart disease was not. This phenomenon is likely due to both inadequate statistical power and to the low risk of coronary heart disease in Japanese women. Some reports indicate a higher risk of stroke in women [37, 38]. Di Tullio et al [38] have shown that smaller aortic plaques are significantly associated with ischemic stroke in women but not in men. This gender difference may be a consequence of the effects of hypercoagulable states [39, 40], lipid abnormalities [41], or gonadal steroids [42–44] in women. Further studies are necessary to elucidate these gender differences in detail.

Several limitations of our study should be discussed. The primary limitation is the small numbers of both subjects and cardiovascular disease events in the study population. Thus, the generalizability of the study results may be somewhat limited. Nonetheless, we believe that the findings of our study represent the actual association between chronic kidney disease and cardiovascular disease outcomes, since we used a highly accurate method of determining all cardiovascular disease cases.

The second limitation is that our results might be biased, because almost 20% of the target population did not participate. At baseline, the mean age of subjects who did not participate was significantly lower than that of subjects who did participate (53 vs. 60 years old), and the proportion of men was significantly higher among nonparticipants (57% vs. 42%). Unfortunately, we could not obtain information on other risk factors among the nonparticipants. However, it is generally agreed that an acceptable participation rate in a population-based study (i.e., a rate that practically eliminates the threat of selection bias attributable to nonparticipants) is above 70% of the target population [45, 46]. Because of the high participation rate in our study (81%), this bias did not seem to have the potential to alter our findings.

The third limitation is that our GFR estimates, which were made using the simplified prediction equation derived from the MDRD Study and that were based on a single blood sample, might not be sufficiently correct, although this prediction equation, among other equations of its type, is considered to be the most precise estimate of GFR [28]. In addition, a recent report has shown that repeated measurements of serum creatinine are necessary to correct within-person measurement variations of serum creatinine [47], suggesting that some nondifferential misclassifications of cases with chronic kidney disease may have occurred in our study. Given that this limitation can reduce the impact of chronic kidney disease, the true association may be stronger than that shown in our findings.

The fourth limitation is that we have no information regarding the severity or duration of hypertension or other cardiovascular disease risk factors. The fifth limitation is that we also could not provide information regarding the type or number of antihypertensive drugs, medication compliance, and blood pressure control. Although ECG abnormalities, which reflect target-organ damage from hypertension or other risk factors, were used as a confounding factor in the multivariate analysis, these limitations may reduce the accuracy of our findings to some extent. Thus, they have the potential to alter our findings, but they are not likely to do so.

The sixth limitation is that our subjects with chronic kidney disease may have undergone more intense medical surveillance than those without it, resulting in a surveillance bias. However, diagnostic procedures such as echocardiography and scintigraphy were usually performed in subjects who presented symptoms or clinical signs of cardiac ischemia, but were not performed in subjects who did not present cardiac symptoms, even if they had chronic kidney disease. Brain CT/MRI was taken in the similar situation. In addition, as described in the **Methods** section, the diagnosis of cardiovascular disease was in principle based on the acute events of heart and brain attack. We performed almost the same follow-up surveys on all study subjects regardless of the presence or absence of chronic kidney disease. The mean number of health investigations was similar for subjects with or without chronic kidney disease in men ($4 \pm 4$ times in subjects with chronic kidney disease vs. $5 \pm 4$ times in subjects without chronic kidney disease) and in women ($5 \pm 4$ times vs. $6 \pm 4$ times). Furthermore, health status was checked yearly by mail or telephone for any subjects who did not undergo a regular examination that year or who had moved out of town. Thus, subjects with chronic kidney disease are considered not to have undergone more intense medical surveillance, so the potential for such bias seems to be negligible.

## CONCLUSION

Chronic kidney disease was found to be an independent risk factor for the incidence of cardiovascular disease in a general Japanese population. Our findings suggest that subjects with chronic kidney disease should be considered a high-risk population for cardiovascular disease and be recommended for more intensive preventive management of cardiovascular disease, including active detection and strict treatment of cardiovascular risk factors. An additional clinical intervention trial is needed to evaluate preventive measures of cardiovascular disease in subjects with chronic kidney disease.

*Reprint requests to Toshiharu Ninomiya, M.D., Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582 Japan.*
*E-mail: nino@intmed2.med.kyushu-u.ac.jp*

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# Relationship Between Drinking and Periodontitis: The Hisayama Study

Yoshihiro Shimazaki,* Toshiyuki Saito,* Yutaka Kiyohara,† Isao Kato,† Michiaki Kubo,† Mitsuo Iida,† and Yoshihisa Yamashita*

**Background:** Although recent studies suggest a relationship between alcohol consumption and periodontal disease, the dose-response relationship between drinking and the severity of periodontitis is unclear.

**Methods:** Alcohol consumption was evaluated using the frequency of drinking and the daily alcohol intake for 961 individuals aged 40 to 79 years. Periodontal status was evaluated using probing depth (PD) and clinical attachment loss (CAL).

**Results:** Alcohol consumption was linearly associated with the extent of PD and CAL in univariate analyses ($P<0.001$). In multivariate logistic regression analyses, the subjects drinking 15 to 29.9 g alcohol per day (odds ratio [OR] = 2.7; 95% confidence interval [CI] = 1.1 to 6.6) or more than 30 g per day (OR = 2.5; 95% CI = 1.1 to 5.7) had a significantly higher risk of having more than 35% of their teeth with PD ≥4 mm than non-drinkers, independent of other confounding variables. No significant relationship between drinking and CAL was observed in the multivariate analysis.

**Conclusion:** These results suggest that the effect of drinking on periodontal condition is limited to subjects with deep periodontal pockets associated with more than one-third of their teeth. *J Periodontol 2005;76:1534-1541.*

**KEY WORDS**

**Alcoholic beverages/adverse effects; periodontitis/epidemiology; risk factors.**

* Department of Preventive Dentistry, Kyushu University Faculty of Dental Science, Fukuoka, Japan.
† Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University.

Both smoking and drinking are lifestyle factors that cause health problems. Numerous studies have shown a relationship between smoking and periodontitis,[1-3] while there is very limited information about the relationship between drinking and periodontitis.[4-7] Previous studies examined the relationship between drinking and probing depth (PD)[4] or clinical attachment loss (CAL).[5] Pitiphat et al. reported a longitudinal relationship between drinking and self-reported periodontitis.[6] Recently, Nishida et al. reported that alcohol consumption is a risk indicator in subjects with the aldehyde dehydrogenase-2 ($ALDH_2$) *1/*2 genotype, but not in subjects with $ALDH_2$ *1/*1 genotype.[7] However, these studies did not find a dose-response relationship between drinking and the severity of periodontitis or conclude whether drinking has a greater effect on PD or CAL.

Drinking also affects several systemic diseases in adults, and many studies have reported J- or U-shaped associations, in which light or moderate alcohol consumption lowers the risk of hypertension,[8,9] coronary heart disease,[10,11] systemic markers of inflammation,[12] and mortality.[13] However, there are no reports on the effect of a low alcohol intake on periodontal disease. In this study, we examined the dose-response relationship between drinking and various stages of periodontal condition and examined how alcohol intake is related to periodontal condition using the results of a health examination conducted in Hisayama.

## MATERIALS AND METHODS

### *Study Population*

The Hisayama Study began in 1961 and is an ongoing population based prospective cohort study of cardiovascular diseases. The town population, based on data from national census, was shown to be representative of Japan as a whole.[14] As a part of the study, from July to September 1998, 982 Hisayama residents aged 40 to 79 years (21.6% of the total population in that age group) underwent a comprehensive health examination, including a dental examination. We excluded 21 subjects who had less than 10 teeth or lacked data for the variables studied; consequently we analyzed 961 subjects (378 male, 583 female) in this study. The Ethics Committee of Kyushu University Faculty of Dental Science and the Department of General Affairs and Health and Welfare of Hisayama approved the study design, data collection methods, and procedure for obtaining informed consent.

### *Oral Examination*

The periodontal examination followed the method of the Third National Health and Nutrition Examination Survey (NHANES III).[15] As periodontal parameters, PD and CAL were measured at mesio-buccal and mid-buccal sites for all of the teeth present in two randomly selected quadrants: one maxillary and one mandibular. We divided the subjects into four categories according to the proportion of teeth with PD ≥4 mm. None: no teeth with PD ≥4 mm; low: 0.1% to 19.9% teeth with PD ≥4 mm; mid: 20% to 34.9% teeth with PD ≥4 mm (the second highest 10th percentile); and high: ≥35% teeth with PD ≥4 mm (the highest 10th percentile). Similarly, the proportion of teeth with CAL ≥5 mm was categorized into four categories. None: no teeth with CAL ≥5 mm; low: 0.1% to 9.9% teeth with CAL ≥5 mm; mid: 10% to 21.9% teeth with CAL ≥5 mm (the second highest 10th percentile); and high: ≥22% teeth with CAL ≥5 mm (the highest 10th percentile). Oral hygiene status was evaluated using the plaque index[16] and we used the mean score of each subject in the analyses.

### *General Examination*

A self-administered questionnaire was completed in advance and checked by trained nurses. Participants answered items concerning their frequency of alcohol intake over the previous year and the kinds and amounts of alcoholic beverages habitually consumed. The alcohol intake per drink was converted into the weight of 100% ethanol in grams. The estimated alcohol content was 21.5 g for a cup of Japanese sake (180 ml), 22.6 g for a bottle of beer (633 ml), 35.7 g for a cup of distilled spirits (180 ml), and 31.8 g for a glass of whiskey (100 ml). The daily amount of drinking was estimated by multiplying the frequency of consuming each drink per week by the weight of ethanol in each drink and dividing the sum by seven (g/day). The daily amount of drinking was divided into four categories: non-drinker (0 g/day), light drinker (0.1 to 14.9 g/day), moderate drinker (15 to 29.9 g/day), and heavy drinker (≥30 g/day). As the number of former drinkers was very low (N = 27; 2.8%), we included past drinkers with non-drinkers. The amount of smoking, including past smoking, was given as the number of cigarettes smoked per day multiplied by the total years of smoking. The amount of smoking was divided into four categories: never smoked, light smoker (1 to 399), moderate smoker (400 to 799), and heavy smoker (≥800). Blood samples were collected from an antecubital vein after an overnight fast. Laboratory analyses of the blood samples followed previously described methods.[17] A 75 g oral glucose tolerance test was performed between 8:00 a.m. and 10:30 a.m. Before and 120 minutes after ingesting the 75 g glucose solution, blood samples were obtained for laboratory measurements. The glucose tolerance was categorized into three groups: normal (fasting and 2-hour post-challenge plasma glucose levels <110 and <140 mg/dl, respectively), diabetes (levels ≥126 or ≥200 mg/dl, respectively), and impaired (other than normal or diabetes).

### *Statistical Analysis*

The differences in percentages were evaluated using Pearson's chi square test and its linearity was evaluated using the Mantel-Haenszel chi square test. The differences in the mean values were evaluated using Student *t* test. To protect against spurious significance with multiple inference, we used Bonferroni's correction to interpret the significance of *P* value. We performed univariate and multivariate logistic regression analyses to determine the effect of alcohol consumption on periodontal parameters, and calculated the odds ratio (OR) and 95% confidence interval (CI). As both PD and CAL were classified into four categories, we performed three logistic regression models using none versus each of the other three categories (low, mid, and high) as the dependent variable. Multivariate models were adjusted for amount of smoking, glucose tolerance, age, sex, number of teeth, and mean plaque index. The statistical analysis was performed using a software program.‡

## RESULTS

Tables 1 and 2 show the characteristics of the subjects according to the proportion of teeth with PD ≥4 mm and with CAL ≥5 mm, respectively. The more alcohol the subjects consumed, the greater the proportion of their teeth with PD ≥4 mm and CAL ≥5 mm,

‡ Version 11.0, SPSS Japan, Tokyo, Japan.

**Table 1.**

## Study Population Variables According to Periodontal Status (PD)

| Variable | Teeth With PD ≥4 mm: None 549 (57.1%) | Low 220 (22.9%) | Mid 102 (10.6%) | High 90 (9.4%) | P Value |
|---|---|---|---|---|---|
| | N (%) | | | | |
| Alcohol consumption | | | | | |
| None (0 g/day) | 355 (60.1) | 126 (21.3) | 67 (11.3) | 43 (7.3) | <0.001* |
| Light (0.1-14.9 g/day) | 91 (59.1) | 41 (26.6) | 14 (9.1) | 8 (5.2) | <0.001† |
| Moderate (15-29.9 g/day) | 46 (50.0) | 24 (26.1) | 7 (7.6) | 15 (16.3) | |
| Heavy (≥30 g/day) | 57 (46.0) | 29 (23.4) | 14 (11.3) | 24 (19.4) | |
| Smoking | | | | | |
| Never (0) | 400 (62.3) | 137 (21.3) | 59 (9.2) | 46 (7.2) | <0.001* |
| Light (1-399) | 47 (52.8) | 27 (30.3) | 8 (9.0) | 7 (7.9) | <0.001† |
| Moderate (400-799) | 63 (51.6) | 31 (25.4) | 12 (9.8) | 16 (13.1) | |
| Heavy (≥800) | 39 (36.1) | 25 (23.1) | 23 (21.3) | 21 (19.4) | |
| Glucose tolerance | | | | | |
| Normal | 404 (60.3) | 145 (21.6) | 67 (10.0) | 54 (8.1) | 0.065* |
| Impaired | 97 (50.8) | 51 (26.7) | 20 (10.5) | 23 (12.0) | 0.002† |
| Diabetes | 48 (48.0) | 24 (24.0) | 15 (15.0) | 13 (13.0) | |
| Gender | | | | | |
| Male | 189 (50.0) | 94 (24.9) | 46 (12.2) | 49 (13.0) | 0.001* |
| Female | 360 (61.7) | 126 (21.6) | 56 (9.6) | 41 (7.0) | <0.001† |
| | Mean ± SD | | | | |
| Age | 55.6 ± 8.7 | 57.3 ± 8.5 | 59.0 ± 8.5§ | 55.6 ± 8.7 | |
| Number teeth | 25.6 ± 3.7 | 25.0 ± 3.5 | 23.7 ± 4.9§ | 23.7 ± 4.6§ | |
| Mean plaque index | 1.0 ± 0.5 | 1.1 ± 0.6‡ | 1.4 ± 0.7§‖ | 1.6 ± 0.7§‖ | |

* Non-linear component calculated using Pearson's chi square test.
† Linear component calculated using Mantel-Haenszel chi square test.
‡ $P<0.05$ compared with none; Student $t$ test applied with Bonferroni's correction of $P$ value for type 1 errors in multiple comparisons.
§ $P<0.01$ compared with none; Student $t$ test applied with Bonferroni's correction of $P$ value for type 1 errors in multiple comparisons.
‖ $P<0.01$ compared with low; Student $t$ test applied with Bonferroni's correction of $P$ value for type 1 errors in multiple comparisons.

which was the same as when the subjects consumed more cigarettes. The subjects with poor diabetic conditions had more teeth with PD ≥4 mm and CAL ≥5 mm. The variables age, gender, number of teeth, and mean plaque index were each significantly associated with the proportion of teeth with PD ≥4 mm and CAL ≥5 mm in the univariate analyses (Tables 1 and 2).

Table 3 shows the univariate and multivariate logistic regression analyses for each of the three different PD conditions. Alcohol consumption did not show any significant influence for having the low or mid PD condition. However, moderate and heavy drinkers had a significantly high OR for having a high proportion of teeth with PD ≥4 mm in the univariate and multivariate analysis adjusting for confounding variables. In the analysis, heavy smoking and a higher plaque index also had a significantly increased OR for having the high PD condition. Table 4 (pages 1540 and 1541) shows the univariate and multivariate ORs for each of the three CAL conditions. Although moderate and heavy drinking had a significantly increased OR for having a high proportion of CAL ≥5 mm in the univariate analysis, the relationship disappeared after multivariate adjustment. Moderate and heavy smoking were associated with significantly increased OR for high CAL, and heavy smoking had a significantly increased OR for low CAL.

## DISCUSSION

This study showed that subjects who drank more than 15 g alcohol per day had a significantly increased risk for widespread periodontal disease; i.e., more than one third of teeth with PD ≥4 mm, as compared to non-drinkers. Conversely, drinking did not indicate an increased risk for having less than 35% of the teeth with PD ≥4 mm. It was reported that the subjects with $ALDH_2$ *1/*2 genotype who consumed ≥33 g alcohol per day had a significantly greater percentage of PD ≥3.5 mm than those whose daily consumption was lower, while there was no significant difference in periodontal status associated with alcohol consumption in $ALDH_2$ *1/*1 subjects.[7] The subjects with $ALDH_2$ genotypes *1/*2 or *2/*2 lack $ALDH_2$ activity and become flushed after alcohol intake owing to the marked elevation in the blood acetaldehyde concentration.[18] Therefore, it is thought that drinking raises the risk of periodontitis when drinking causes an accumulation of acetaldehyde. As about half of all Japanese lack $ALDH_2$ activity,[19,20] many subjects with $ALDH_2$ *1/*2 genotype might have been included in the subjects with many teeth with PD ≥4 mm in our study.

Periodontitis is a chronic inflammatory disease of the soft and hard periodontal tissues and recent studies have suggested a relationship between periodontitis and circulatory diseases.[21-23] Inflammation plays an important role in both the initiation and pro-

**Table 2.**

## Study Population According to Periodontal Status (CAL)

| Variable | Teeth With CAL ≥5 mm: None | Low | Mid | High | *P* Value |
|---|---|---|---|---|---|
| | 624 (64.9%) | 146 (15.2%) | 95 (9.9%) | 96 (10.0%) | |
| | N (%) | | | | |
| Alcohol consumption | | | | | |
| None (0 g/day) | 394 (66.7) | 85 (14.4) | 65 (11.0) | 47 (8.0) | 0.002* |
| Light (0.1-14.9 g/day) | 106 (68.8) | 26 (16.9) | 12 (7.8) | 10 (6.5) | <0.001† |
| Moderate (15-29.9 g/day) | 57 (62.0) | 12 (13.0) | 8 (8.7) | 15 (16.3) | |
| Heavy (≥30 g/day) | 67 (54.0) | 23 (18.5) | 10 (8.1) | 24 (19.4) | |
| Smoking | | | | | |
| Never (0) | 458 (71.3) | 84 (13.1) | 62 (9.7) | 38 (5.9) | <0.001* |
| Light (1-399) | 53 (59.6) | 18 (20.2) | 10 (11.2) | 8 (9.0) | <0.001† |
| Moderate (400-799) | 70 (57.4) | 20 (16.4) | 14 (11.5) | 18 (14.8) | |
| Heavy (≥800) | 43 (39.8) | 24 (22.2) | 9 (8.3) | 32 (29.6) | |
| Glucose tolerance | | | | | |
| Normal | 461 (68.8) | 90 (13.4) | 59 (8.8) | 60 (9.0) | <0.001* |
| Impaired | 114 (59.7) | 36 (18.8) | 27 (14.1) | 14 (7.3) | <0.001† |
| Diabetes | 49 (49.0) | 20 (20.0) | 9 (9.0) | 22 (22.0) | |
| Gender | | | | | |
| Male | 206 (54.5) | 69 (18.3) | 42 (11.1) | 61 (16.1) | <0.001* |
| Female | 418 (71.7) | 77 (13.2) | 53 (9.1) | 35 (6.0) | <0.001† |
| | Mean ± SD | | | | |
| Age | 55.0 ± 8.6 | 57.7 ± 8.3‡ | 59.7 ± 8.2‡ | 59.9 ± 9.1‡ | |
| Number of teeth | 25.7 ± 3.8 | 25.9 ± 2.7 | 23.5 ± 4.4‡§ | 22.0 ± 4.7‡§ | |
| Mean plaque index | 1.0 ± 0.6 | 1.1 ± 0.6 | 1.2 ± 0.6‡ | 1.6 ± 0.6‡§‖ | |

* Non-linear component calculated using Pearson's chi square test.
† Linear component calculated using Mantel-Haenszel chi square test.
‡ $P < 0.01$ compared with none; Student *t* test applied with Bonferroni's correction of *P* value for type 1 errors in multiple comparisons.
§ $P < 0.01$ compared with low; Student *t* test applied with Bonferroni's correction of *P* value for type 1 errors in multiple comparisons.
‖ $P < 0.01$ compared with mid; Student *t* test applied with Bonferroni's correction of *P* value for type 1 errors in multiple comparisons.

gression of atherosclerosis,[24] and the systemic inflammatory marker such as C-reactive protein (CRP) is a predictor of cardiovascular events.[25] The subjects with periodontitis had a higher CRP level than the subjects with healthy periodontal tissue.[26,27] Periodontal disease was significantly associated with a higher CRP level in a longitudinal study[28] and recent studies reported that control of periodontal health decreased the serum CRP level.[29,30] Although CRP level is unknown in this study, as our results showed that moderate to heavy drinking was associated with a significant risk of having many teeth with deep PD, increased periodontal inflammation with alcohol consumption may increase the risk of coronary heart disease, in addition to the direct effect of alcohol on the circulatory system.

Tezal et al. reported a significant relationship between the frequency of drinking and CAL.[5] We did not find a significant relationship between drinking and CAL. It may be owing to small sample size, especially the low number of drinkers in this study. Alcohol is considered an important risk factor for various bone-related disorders, such as reduced bone mass and fractures, and chronic alcohol abuse is a major risk factor for osteoporosis.[31,32] A 2001 study found a relationship between osteoporosis and periodontitis in menopausal women.[33] If drinking exacerbates alveolar bone resorption, the observed effect of drinking on increasing periodontal pocket depth may lead to extensive periodontal destruction.

Some studies have reported J- or U-shaped relationship in which light drinkers had a lower risk of hypertension, coronary heart disease, systemic markers of inflammation, and mortality of all causes than did non-drinkers or heavy drinkers.[8-13] Previous studies of the relationship between drinking and periodontitis failed to find a significant association between light drinking and periodintitis, although two studies showed that light drinkers tended to have better periodontal health than non-drinkers.[5,7] In our study, although light drinkers had a relatively low risk for having many teeth with deep PD, the relationship was not significant statistically. It is thought that a large number of study subjects is needed to clarify the effect of light drinking on periodontitis.

Smoking is an important lifestyle-related risk factor for periodontitis, and this study suggests that heavy drinking is also a risk factor for periodontitis. Smoking cessation should be strongly recommended for patients with periodontitis. As our results were based on a cross-sectional investigation, we could not clarify causal relationship between drinking and periodontitis. Therefore, at this stage, we may advise heavy drinkers with periodontitis to reduce the amount they drink to improve both their systemic and oral health. In order to establish the

**Table 3.**

## Risk for Low, Mid, and High Proportion of Teeth With PD ≥4 mm According to Alcohol Consumption and Other Variables

| | Model 1 | | | | Model 2 | | | |
|---|---|---|---|---|---|---|---|---|
| Independent Variable | Teeth With PD ≥4 mm: None | Low | Univariate OR (95% CI) | Multivariate OR (95% CI) | Teeth With PD ≥4 mm: None | Mid | Univariate OR (95% CI) | Multivariate OR (95% CI) |
| Alcohol consumption | | | | | | | | |
| None (0 g/day) | 355 | 126 | 1 | 1 | 355 | 67 | 1 | 1 |
| Light (0.1-14.9 g/day) | 91 | 41 | 1.3 (0.8-1.9) | 1.3 (0.8-2.0) | 91 | 14 | 0.8 (0.4-1.5) | 0.7 (0.4-1.5) |
| Moderate (15-29.9 g/day) | 46 | 24 | 1.5 (0.9-2.5) | 1.3 (0.7-2.4) | 46 | 7 | 0.8 (0.3-1.9) | 0.8 (0.3-2.0) |
| Heavy (≥30 g/day) | 57 | 29 | 1.4 (0.9-2.4) | 1.1 (0.6-2.0) | 57 | 14 | 1.3 (0.7-2.5) | 0.7 (0.3-1.7) |
| Smoking | | | | | | | | |
| Never (0) | 400 | 137 | 1 | 1 | 400 | 59 | 1 | 1 |
| Light (1-399) | 47 | 27 | 1.7 (1.0-2.8)* | 1.7 (0.9-3.1) | 47 | 8 | 1.2 (0.5-2.6) | 1.4 (0.6-3.7) |
| Moderate (400-799) | 63 | 31 | 1.4 (0.9-2.3) | 1.4 (0.7-2.5) | 63 | 12 | 1.3 (0.7-2.5) | 1.4 (0.6-3.5) |
| Heavy (≥800) | 39 | 25 | 1.9 (1.1-3.2)* | 1.6 (0.8-3.2) | 39 | 23 | 4.0 (2.2-7.2)† | 3.5 (1.4-8.7)† |
| Glucose tolerance | | | | | | | | |
| Normal | 404 | 145 | 1 | 1 | 404 | 67 | 1 | 1 |
| Impaired | 97 | 51 | 1.5 (1.0-2.2) | 1.4 (0.9-2.0) | 97 | 20 | 1.2 (0.7-2.1) | 1.0 (0.6-1.8) |
| Diabetes | 48 | 24 | 1.4 (0.8-2.4) | 1.1 (0.7-2.0) | 48 | 15 | 1.9 (1.0-3.6) | 1.5 (0.7-3.0) |
| Gender | | | | | | | | |
| Male | 189 | 94 | 1 | 1 | 189 | 46 | 1 | 1 |
| Female | 360 | 126 | 0.7 (0.5-1.0)* | 1.1 (0.6-1.8) | 360 | 56 | 0.6 (0.4-1.0)* | 1.2 (0.6-2.7) |
| Age (years) | | | 1.0 (1.0-1.0)* | 1.0 (1.0-1.0) | | | 1.0 (1.0-1.1)‡ | 1.0 (1.0-1.0) |
| Number of teeth | | | 1.0 (0.9-1.0)* | 1.0 (0.9-1.0) | | | 0.9 (0.9-0.9)‡ | 1.0 (0.9-1.0) |
| Mean plaque index | | | 1.5 (1.1-2.0)† | 1.3 (1.0-1.7) | | | 3.6 (2.5-5.2)‡ | 3.0 (2.0-4.4)‡ |

* $P<0.05$.
† $P<0.01$.
‡ $P<0.001$.

effect of drinking as a risk factor for periodontitis, larger-scale epidemiological and interventional studies, for example examining the effect of temperance and abstinence from drinking in heavy drinkers with periodontitis, are needed to confirm the causal relationship between drinking and periodontitis, as well as supportive experimental studies to clarify the mechanisms for the relationship between drinking and periodontitis.

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**Table 3. (continued)**

**Risk for Low, Mid, and High Proportion of Teeth With PD ≥4 mm According to Alcohol Consumption and Other Variables**

| | Model 3 | | |
|---|---|---|---|
| | Teeth With PD ≥4 mm | | |
| | None | High | Univariate OR (95% CI) | Multivariate OR (95% CI) |
| | 355 | 43 | 1 | 1 |
| | 91 | 8 | 0.7 (0.3-1.6) | 0.6 (0.3-1.6) |
| | 46 | 15 | 2.7 (1.4-5.2)† | 2.7 (1.1-6.6)* |
| | 57 | 24 | 3.5 (2.0-6.2)† | 2.5 (1.1-5.7)* |
| | 400 | 46 | 1 | 1 |
| | 47 | 7 | 1.3 (0.6-3.0) | 1.2 (0.4-3.2) |
| | 63 | 16 | 2.2 (1.2-4.1)* | 1.7 (0.7-4.2) |
| | 39 | 21 | 4.7 (2.5-8.6)† | 2.8 (1.1-7.3)* |
| | 404 | 54 | 1 | 1 |
| | 97 | 23 | 1.8 (1.0-3.0)* | 1.2 (0.7-2.3) |
| | 48 | 13 | 2.0 (1.0-4.0)* | 1.3 (0.6-3.0) |
| | 189 | 49 | 1 | 1 |
| | 360 | 41 | 0.4 (0.3-0.7)† | 1.7 (0.7-3.9) |
| | | | 1.0 (1.0-1.0) | 1.0 (0.9-1.0) |
| | | | 0.9 (0.9-0.9)‡ | 0.9 (0.9-1.0) |
| | | | 5.4 (3.6-8.0)‡ | 4.6 (3.0-7.0)‡ |


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**Table 4.**

## Risk for Low, Mid, and High Proportion of Teeth With CAL ≥5 mm According to Alcohol Consumption and Other Variables

| | Model 1 | | | | Model 2 | | | |
|---|---|---|---|---|---|---|---|---|
| Independent Variable | Teeth With CAL ≥5 mm | | Univariate OR (95% CI) | Multivariate OR (95% CI) | Teeth With CAL ≥5 mm | | Univariate OR (95% CI) | Multivariate OR (95% CI) |
| | None | Low | | | None | Mid | | |
| Alcohol consumption | | | | | | | | |
| None (0 g/day) | 394 | 85 | 1 | 1 | 394 | 65 | 1 | 1 |
| Light (0.1-14.9 g/day) | 106 | 26 | 1.1 (0.7-1.9) | 1.0 (0.6-1.7) | 106 | 12 | 0.7 (0.4-1.3) | 0.6 (0.3-1.3) |
| Moderate (15-29.9 g/day) | 57 | 12 | 1.0 (0.5-1.9) | 0.7 (0.3-1.5) | 57 | 8 | 0.9 (0.4-1.9) | 0.6 (0.2-1.4) |
| Heavy (≥30 g/day) | 67 | 23 | 1.6 (0.9-2.7) | 0.9 (0.5-1.8) | 67 | 10 | 0.9 (0.4-1.8) | 0.5 (0.2-1.1) |
| Smoking | | | | | | | | |
| Never (0) | 458 | 84 | 1 | 1 | 458 | 62 | 1 | 1 |
| Light (1-399) | 53 | 18 | 1.9 (1.0-3.3)* | 2.0 (1.0-3.9) | 53 | 10 | 1.4 (0.7-2.9) | 1.5 (0.6-3.5) |
| Moderate (400-799) | 70 | 20 | 1.6 (0.9-2.7) | 1.5 (0.8-3.1) | 70 | 14 | 1.5 (0.8-2.8) | 1.1 (0.5-2.6) |
| Heavy (≥800) | 43 | 24 | 3.0 (1.8-5.3)‡ | 2.6 (1.3-5.4)* | 43 | 9 | 1.5 (0.7-3.3) | 0.9 (0.3-2.3) |
| Glucose tolerance | | | | | | | | |
| Normal | 461 | 90 | 1 | 1 | 461 | 59 | 1 | 1 |
| Impaired | 114 | 36 | 1.6 (1.0-2.5)* | 1.5 (0.9-2.3) | 114 | 27 | 1.9 (1.1-3.0)* | 1.7 (1.0-2.9)* |
| Diabetes | 49 | 20 | 2.1 (1.2-3.7)* | 1.7 (0.9-3.1) | 49 | 9 | 1.4 (0.7-3.1) | 0.9 (0.4-2.0) |
| Gender | | | | | | | | |
| Male | 206 | 69 | 1 | 1 | 206 | 42 | 1 | 1 |
| Female | 418 | 77 | 0.6 (0.4-0.8)† | 1.0 (0.5-1.7) | 418 | 53 | 0.6 (0.4-1.0)† | 0.5 (0.2-0.9)* |
| Age (years) | | | 1.0 (1.0-1.1)† | 1.0 (1.0-1.1)† | | | 1.1 (1.0-1.1)† | 1.0 (1.0-1.1)† |
| Number teeth | | | 1.0 (1.0-1.1) | 1.1 (1.0-1.1)* | | | 0.9 (0.8-0.9)‡ | 0.9 (0.9-1.0)† |
| Mean plaque index | | | 1.4 (1.0-1.9)* | 1.3 (0.9-1.7) | | | 1.8 (1.3-2.6)‡ | 1.3 (0.8-1.8) |

* $P<0.05$.
† $P<0.01$.
‡ $P<0.001$.

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Correspondence: Dr. Yoshihiro Shimazaki, Department of Preventive Dentistry, Kyushu University Faculty of Dental Science, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Fax: 81-92-642-6354; e-mail: shimadha@mbox.nc.kyushu-u.ac.jp.

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**Table 4. (continued)**

## Risk for Low, Mid, and High Proportion of Teeth With CAL ≥5 mm According to Alcohol Consumption and Other Variables

| | | | Model 3 | |
|---|---|---|---|---|
| | Teeth With CAL ≥5 mm | | | |
| | None | High | Univariate OR (95% CI) | Multivariate OR (95% CI) |
| | 394 | 47 | 1 | 1 |
| | 106 | 10 | 0.8 (0.4-1.6) | 0.6 (0.3-1.4) |
| | 57 | 15 | 2.2 (1.2-4.2)† | 1.4 (0.6-3.3) |
| | 67 | 24 | 3.0 (1.7-5.2)‡ | 1.2 (0.5-2.6) |
| | 458 | 38 | 1 | 1 |
| | 53 | 8 | 1.8 (0.8-4.1) | 1.9 (0.7-5.1) |
| | 70 | 18 | 3.1 (1.7-5.7)‡ | 2.8 (1.2-6.8)* |
| | 43 | 32 | 9.0 (5.1-15.8)‡ | 4.9 (1.9-12.2)† |
| | 461 | 60 | 1 | 1 |
| | 114 | 14 | 0.9 (0.5-1.7) | 0.6 (0.3-1.1) |
| | 49 | 22 | 3.5 (2.0-6.1)‡ | 2.0 (1.0-3.9)* |
| | 206 | 61 | 1 | 1 |
| | 418 | 35 | 0.3 (0.2-0.4)‡ | 1.0 (0.4-2.3) |
| | | | 1.1 (1.0-1.1)† | 1.0 (1.0-1.1) |
| | | | 0.8 (0.8-0.9)‡ | 0.9 (0.8-1.0)† |
| | | | 4.3 (3.0-6.2)‡ | 2.9 (1.9-4.4)‡ |

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# Characterization of the Structure and Expression of Mouse *Itpa* Gene and its Related Sequences in the Mouse Genome

Mehrdad BEHMANESH,[1,2] Kunihiko SAKUMI,[1] Daisuke TSUCHIMOTO,[1] Kumiko TORISU,[1] Yoko OHNISHI-HONDA,[1] Derrick E. RANCOURT,[3] and Yusaku NAKABEPPU[1,*]

*Division of Neurofunctional Genomics, Department of Immunobiology and Neuroscience, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan,[1] Department of Genetics, School of Sciences, Tarbiat Modarres University, Tehran, Iran,[2] and Southern Alberta Cancer Research Center, Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Alberta, Canada, T2N 4N1[3]*

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**Abstract**

In the mouse genome, we found one processed *Itpa* gene-like sequence and two processed *Itpa* pseudogenes as well as the *Itpa* gene itself with introns, located on chromosome 2F3, which was isolated by a retro-recombination method. We also identified three types (A, B, C) of *Itpa* transcripts in mouse tissues. The processed *Itpa* gene-like sequence located on chromosome 2E1 has a complete open reading frame for exactly the same polypeptide as ITPA encoded by the type A transcript, with a polyadenylation signal. However, no transcribed sequence derived from the *Itpa* gene-like sequence was detectable in any of the mouse tissues examined, thus naming the sequence as *Itpa* processed pseudogene $\alpha$. The type A *Itpa* mRNA, which was expressed in all mouse tissues examined, only encodes mouse ITPA polypeptide consisting of 198 amino acid residues with a capacity to hydrolyze dITP into dIMP. *Itpa* mRNA was detected in all tissues examined, and its expression is especially high in the testis, brain, and thymus. ITPA protein was mostly detected in the cytoplasm, to a lesser extent in the nuclei of neurons in the brain, and also those of hepatocytes, epithelial cells lining the bile duct, and endothelial cells lining the portal vein in the liver.

**Key words:** ITPase; ITP; XTP; pseudogene; retro-recombination; oxidative deamination

## 1. Introduction

The accumulation of modified or damaged bases in genomic DNA is a major cause of altered genetic information that results in mutagenesis or even programmed cell death.[1] It has been established that such damaged bases in genomic DNA arise from two independent pathways: one is a consequence of the direct modification of the normal bases in the DNA and the other is incorporation of modified nucleotides generated in resident nucleotide pools.[2,3]

The incorporation of dUTP into DNA which arises from the deamination of dCTP or as a byproduct of UTP biosynthesis is a major cause for shortening the Okazaki fragment during nascent strand synthesis that occurs during base excision repair by uracil DNA glycosylase.[4] dUTP is thus detrimental to organisms and it is eliminated by a specific nucleotidase, dUTPase, from the resident nucleotide pools.[5–7] 8-Oxo-2′-deoxyguanosine triphosphate (8-oxo-dGTP) is one of the major causes of spontaneous mutagenesis, because 8-oxo-dGTP is formed by the spontaneous oxidation of dGTP in the nucleotide pool and it is incorporated into the nascent strand opposite both adenine and cytosine in the template strand during DNA replication.[8] We have demonstrated that 8-oxo-dGTP is specifically hydrolyzed by MutT family proteins from prokaryotes to humans, and, as a result, organisms maintained a low spontaneous mutation rate.[9–12]

Genome projects revealed the existence of many MutT-like proteins with different substrate specificities,[13–17] and a structure-based approach recently identified another novel enzyme, namely inosine triphosphate pyrophosphatase (ITPase), which hydrolyzes deaminated purine nucleoside triphosphates such as ITP, dITP, and XTP.[18–20] In *Escherichia coli*, a mutant of the *rdgB* gene coding ITPase protein is viable but it shows synthetic lethality with *recA* or *recBC* mutation.[21] Recently, the lethality of *rdgB recA* or *rdgB recBC* double mutants has been shown to be suppressed by the inactivation of endonuclease V (EndoV) which initiates the excision of

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* To whom correspondence should be addressed. Tel. +81 92-642-6800, Fax. +81 92-642-6791, E-mail: yusaku@bioreg.kyushu-u.ac.jp

deoxyinosine (dI) or deoxyxanthosine (dX) incorporated into DNA.[22] It is likely that an ITPase deficiency results in the accumulation of its substrate nucleotides, dITP or dXTP in the nucleotide pools, thus causing an increased accumulation of dI or dX into DNA, and further excision repair initiated by EndoV leads to chromosomal fragmentation in *recA* or *recBC* mutants. Following this study,[22] the missense mutants of the *dut* gene encoding dUTPase, were reported to exhibit synthetic lethality with *recA* or *recBC* mutations, and synthetic lethality was suppressed by inactivation of the *ung* gene encoding uracil DNA glycosylase, thus confirming that the accumulation of abnormal nucleotides in the nucleotide pools increased chromosomal fragmentation as a consequence of the excision repair of such abnormal bases incorporated into DNA.[7]

The first reported deficiency in the ITPase in a human was characterized by an elevated ITP level in erythrocytes as an inherited abnormality.[23] However, such individuals do not exhibit any abnormal phenotype even with accumulation of ITP in erythrocytes. A cDNA for human ITPase was isolated and its gene *ITPA* was identified,[19] and the structure of the *hITPA* gene and nucleotide alterations responsible for the ITPase deficiency were reported.[24,25]

In contrast to *E. coli*, the biological importance of ITPase protein and the pathological consequences of its deficiency in humans or mammals have not yet been elucidated. To explore the biological significance of ITPase in mammals, disruption of the mouse *Itpa* gene and characterization of such mutant mice is considered to be one of the best experimental approaches. During isolation of the *Itpa* gene from the mouse genome, we found one processed *Itpa* gene-like sequence and two processed *Itpa* pseudogenes as well as the *Itpa* gene itself with introns, which was isolated by a retro-recombination method in the present study.

## 2. Materials and Methods

### *2.1. RT-PCR*

Total RNAs from CCE mouse embryonic stem (ES) cells derived from 129SvEv mouse,[26] serum-starved and serum-stimulated BALB/c3T3 cells,[27,28] and C57BL/6J adult mouse tissue specimens were prepared using ISOGEN (Nippon Gene) according to the manufacturer's instructions. Total RNAs from BALB/c mouse tissues were purchased from Clontech. Total RNA (10 $\mu$g) was treated with 20 units of RNase-free deoxyribonuclease I (DNase I) (Amersham Biosciences) at 37°C for 15 min in 100 $\mu$l of reaction buffer containing 40 mM Tris-HCl (pH 7.5) and 6 mM $MgCl_2$. The treated RNA was purified by extraction with Phenol/$CHCl_3$ (1 : 1) followed by ethanol precipitation. The purified RNA (2 $\mu$g) was used for synthesis of the first strand cDNA using a First-strand cDNA synthesis Kit (Amersham Biosciences) according to the manufacturer's instructions. PCR was performed in 20-$\mu$l reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.3 $\mu$l of the first-strand cDNA, 0.4 U of recombinant Taq DNA polymerase (Takara), 4 $\mu$M of each primer (Table 1), and 200 $\mu$M of each deoxynucleoside triphosphate. The initial denaturation was performed at 95°C for 1 min and the amplification was performed by 27, 32, 40 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 20 sec, and extension at 72°C for 60 sec, followed by a final extension at 72°C for 5 min. PCR products were subjected to agarose gel electrophoresis, and the band intensity on the gel stained with ethidium bromide was measured using the LAS1000-plus Luminescent Image Analysis System (FUJI FILM).

**Table 1.** The synthetic oligonucleotides used in this study. These were obtained from Greiner Japan and Hokkaido System Science.

| Oligomer | Seuence |
|---|---|
| mItpa_EX1f | GACGGGAAACGCCAAGAAGCTG |
| mItpa_EX2r | CAAAGTGCATGGAAAATTATCTCCG |
| mItpa_EX2f | TCAATGCACTTTGGAGGCTCAG |
| mItpa_EX3r | TGGAAATCTCATCCGGTTCTCC |
| mItpa_EX3f | CCATACAGAAGTGTCGGGAGGC |
| mItpa_EX4r | AGGTATCTTCCACCAGGACAGG |
| mItpa_EX4f | CTTTAACGCACTTGGGGGACTG |
| mItpa_EX5r | AGGCTTCAGCTTCTGTAGGAAC |
| mItpa_EX5f | AAATGGTTCCTACAGAAGCTGA |
| mItpa_EX6r | GTGCATAGGCCGATTTGTCTTC |
| mItpa_EX6f | CAGCCAGTGCTCCTGTTCAGAG |
| mItpa_EX7r | CGGCTGCCTCGTGGCATCAC |
| mItpa_EX7f | TCCCTGCTTTCAGCCTGATG |
| mItpa_EX8r | TCTTCTCAGACTTTGGCATCTC |
| 3-1mIT | GATATCGCTAGTGGTCACCAGCCCCAG |
| 3-2mIT | GAGCCAACTGCTCTTCCAAGAACTG |
| 3-3mIT | AAGTCTTCTAGAATTTACATTTGC |
| 3-4mIT | CAGACACACCATTTATTTTGGAAA |
| 5-1mIT | *AAGCTTGCC* ATGGCTGCGTCTTTGGTCG[a] |
| 5-3mIT | AAAGGGTTACTGGGCATTCTGG |
| Pα-45 | CCCAATAAGAAACTGGCATCTTTC |
| Pα-46 | AGTTATAGTATAAATAATATCCAAGA |
| 3-1hIT | GAAGTCAAGCTGCCAAACTGC |
| 3-2hIT | CAGACAGGCCGGTGAGGCTACTTG |
| 5-1hIT | GTAACCGGGGATCACCATGGC |
| 5-3hIT | TGGACGCCAAGGAGTTTTCGGTG |

[a]Italic letters indicate a sequence added to create restriction sites for *Hin*dIII and *Nco* I.

### *2.2. Genomic PCR*

Genomic DNA (25 $\mu$g) from CCE cells was treated with RNase A (20 units, Sigma), which was boiled for 15 min to inactivate contaminated DNase, in 150 $\mu$l of a reaction mixture containing 5 mM Tris-HCl (pH 7.6) and

0.5 mM EDTA at 37°C for 30 min to eliminate the residual RNA completely. DNA was purified by extraction with Phenol/$CHCl_3$ followed by ethanol precipitation. A PCR reaction was performed with 100 ng of the purified genomic DNA with appropriate primers (Table 1), as described in the RT-PCR section. The PCR products were subcloned into pT7 Blue T vector (Novagen) plasmid and their nucleotide sequences were confirmed by sequencing the insert from the both strands using ABI Sequencing Kits and a model 3100 automated DNA sequencer (ABI), according to the manufacturer's instructions.

### *2.3. Isolation of genomic clones for* Itpa *gene*

A PCR fragment carrying intron 2 or 3 of *Itpa* amplified from CCE genomic DNA was subcloned into the multi-cloning site of the $\pi$AN$\gamma$ plasmid, and each plasmid was introduced into *E. coli* MC1061[P3] cells.[29] Each cell was infected with $\lambda$ phage genomic library constructed in $\lambda$TK phage with 129 SvJ mouse genomic DNA.[29] Four out of 6 clones isolated with a probe plasmid carrying intron 2, and 8 out of 14 clones isolated with a probe plasmid carrying intron 3, using the retro-recombination methods,[29] contained the appropriate probe sequence and identical adjacent sequences for each other, respectively.

### *2.4. Plasmid construction*

Mouse *Itpa* cDNA was amplified from the mouse testis library (ML 4007AB, Clontech) and from total RNA prepared from CCE cells using 5-3 mIT and 3-3 mIT primers (Table 1). Human *ITPA* cDNA was amplified from a human leukocyte library (HL 4021AB, Clontech) using 5-3 hIT and 3-2 hIT primers (Table 1). PCR products were subcloned into pT7 Blue T Vector. The entire coding region of type A *Itpa* cDNA (615 bp) was amplified with 5-1 mIT and 3-1 mIT primers, in which a *Nco* I site at the initiation site and an *Eco*RV site after the termination codon was introduced, respectively. The PCR product digested with *Nco* I and *Eco*RV was subcloned into a *Nco* I/*Bam*HI site of the pET8c and pET32a plasmids (Novagen), in which a *Bam*HI site was converted to a blunt end by filling in with Klenow fragment. Obtained plasmids were designated pET8c:mITPA and pET32a:mITPA, respectively. A *Nco* I-*Bam*HI fragment of hITPA cDNA was also subcloned into a *Nco* I/*Bam*HI site of pET32a or pET8c plasmid yielding pET32a:hITPA and pET8c:hITPA, respectively.

### *2.5. Immunodetection of* ITPA

Rabbit antiserum against the fusion protein Trx-hITPA were prepared as described previously.[30] The antiserum was able to detect both Trx-hITPA and mITPA proteins with almost the same efficiency, and thus was designated as anti-ITPA. Western blotting analyses were performed as previously described,[31] using anti-ITPA serum (1/500 dilution). Immunohistochemistry with anti-ITPA were performed as follows. The mice were deeply anesthetized with 5% pentobarbital, and perfused transcardially with 10 ml of heparinized saline (0.9%) followed by 30 ml of phosphate buffer (0.1 M) containing 4% paraformaldehyde. Tissue specimens were fixed in 4% paraformaldehyde at 4°C for 12 to 24 hr and embedded in paraffin. The sections (4 $\mu$m) were deparaffinized, pretreated in 3% hydrogen peroxide in methanol, and subjected to immunohistochemistry with anti-ITPA serum (1/500 dilution). The sections were processed by Vectastain ABC KITs (Vector Laboratories) with an anti-rabbit biotinylated secondary antibody, and the peroxidase reaction product was detected using 3,3′-diaminobenzidine-tetrahydrochloride (Sigma). The slides were subjected to counterstaining by hematoxylin. Digital images were acquired using Axioskop2 plus equipped with an AxioCam (Carl Zeiss).

## 3. Results and Discussion

### *3.1. Isolation of Mouse cDNA and Genomic Sequences Homologous to Human* ITPA *cDNA sequence*

We searched the DNA sequence databases to retrieve the mouse cDNA or genome sequences homologous to human *ITPA* cDNA sequence (accession no. AF219116), using the BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/), and found many mouse expressed sequence tags (ESTs) and several mouse genomic sequences. Among the many EST sequences retrieved, the longest cDNA sequence (accession no. AK008279) which encodes a polypeptide highly homologous to the amino acid sequence of human ITPA protein (hITPA) was selected, and it was used for the BLASTN search with the Ensembl Genome Data Resources for mouse (http://www.ensembl.org/Mus_musculus/). The alignment revealed that there are several different mouse chromosomes carrying highly homologous but not identical sequences to that of AK008279 itself, thus suggesting that there are multiple genes for mouse ITPA protein (mITPA) or related proteins.

To isolate cDNA or genomic sequences which encode mITPA, we amplified the sequences from genomic DNA and cDNA prepared from a mouse ES cell line CCE cells which was established from a 129 SvEv mouse, using two different primer sets for the AK008279 EST sequence (Fig. 1A, Table 1). From the RNase A-treated genomic DNA, a major single fragment approximately 1100 bp in length was amplified with primer set I (5-1 mIT and 3-3 mIT), but not with primer set II (5-3 mIT and 3-3 mIT; Fig. 1B, lanes 2, 7). In primer set I, the 5′-primer hybridizes a region including the initiation codon while the 5′ primer in the latter set hybridizes its 5′-untranslated region (UTR). In contrast, two major fragments of approximately 1100 and 830 bp in length from primer set I,

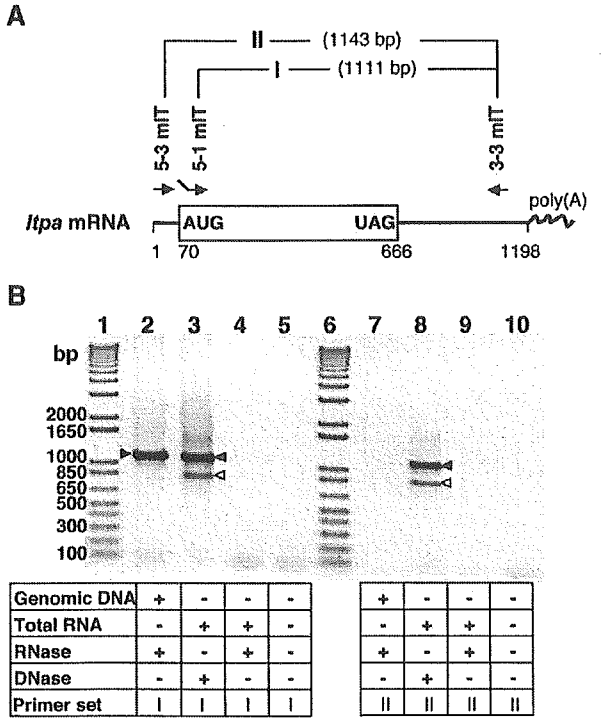



**Figure 1.** Amplification of the mouse *Itpa* cDNA-related sequences from genomic DNA and total RNA prepared from CCE ES cells. *A*. Primer sets for mouse *Itpa* cDNA. Two primer sets (I and II) were designed based on a mouse *Itpa* cDNA (accession no. AK008279). Primer set I with primers 5-1 mIT and 3-3 mIT, in which the 5′-primer hybridizes into the initiation codon with 9-base extra sequence (see Table 1), was expected to amplify the 1111-bp fragment, while primer set II with primers 5-3 mIT and 3-3 mIT, in which the 5′-primer hybridizes into 5′-UTR sequence, was expected to amplify the 1143-bp fragment from *Itpa* cDNA. The coding regions for ITPA and poly(A) sequences are shown with an open box and a wavy line, respectively. *B*. Agarose gel electrophoresis of the PCR products. To avoid cross-contamination of genomic DNA or RNA into total RNA or genomic DNA prepared for PCR templates, genomic DNA was treated with DNase-free RNase A, while total RNA preparation was pre-treated with RNase-free DNase I before cDNA synthesis. PCR was performed with a template and primer set shown under each lane. Lanes 1, 6, size markers. The arrowheads indicate the major PCR products in each reaction: closed, 1108-bp genomic PCR product; gray, 1111- or 1143-bp RT-PCR product; open, 832- or 864-bp RT-PCR product.

or 1140 and 860 bp in length from primer set II were amplified from the cDNA prepared from the DNase I-treated RNA, respectively (Fig. 1B, lanes 3, 8).

A sequence analysis of subcloned PCR products (102 independent clones) obtained by primer set II, revealed three different types of cDNA sequences (types A, B, and C) to be amplified from the RNA prepared from the ES cells (Fig. 2A). In the RT-PCR products, the ratio of each cDNA identified was type A : B : C = 1 : 0.3 : 0.05. The longest type A cDNA isolated was 1143 bp length with an open reading frame (ORF) of 597 nucleotides, whose sequence was identical to AK008279 and was predicted to encode a polypeptide of 198 amino acid residues with a molecular weight of 21,883 Da. The amino acid sequence was 89.9% identical to that of human ITPA (194 aa). Type B and C cDNAs were 864 and 720 bp length, respectively, and had the same 5′- and 3′-UTR sequences as did the type A cDNA. Both of the cDNAs had the same reading frame as that of the type A, but they were shorter than that of type A, and were 318 and 168 nucleotides in length, respectively. Both type B and C cDNA shared the first ATG codon and the stop codon (TAG) with type A cDNA, and were predicted to encode polypeptides with 105 or 55 amino acid residues missing central parts (94–186 aa or 44–186 aa) of the full-length mITPA protein, respectively (Fig. 2A). These results were confirmed by a sequence analysis of RT-PCR products (100 clones) obtained by primer set I, whose length was 1111, 832, or 688 bp, respectively (data not shown).

A sequence analysis of 50 clones of DNA fragments amplified from genomic DNA with primer set I revealed a single 1108-bp fragment whose sequence was 99% identical to that of the type A *Itpa* cDNA (Fig. 2B). The more 5′- and 3′-regions of the genomic sequence were amplified using a new primer set based on the mouse DNA sequence from clone RP23-16O15 on chromosome 2 (accession no. AL672251) which contained exactly the same sequence shown in Fig. 2B (Table 1). The genomic sequence (1135 bp) retained the same ORF of 597 nucleotides which encodes the same polypeptide as does the type A cDNA, with a canonical polyadenylation signal (AATAAA) in the same position as in the type A cDNA. There are three silent nucleotide substitutions in the ORF of the genomic sequence in comparison to that in type A cDNA (Fig. 2B). In addition, one of the five repeats of the CAA triplet found in the 3′-UTR of the type A *Itpa* cDNA sequence was missing in the genomic sequence. These results strongly suggest that there are indeed multiple genomic sequences and transcripts which are capable of encoding mITPA protein or its variants.

### 3.2. *Transcripts for mouse* ITPA *expressed in adult mouse tissue*

Since the genomic sequence amplified with the primer set I has no intron-like sequence with a complete ORF for mITPA protein, it is likely that this genomic sequence is a processed type gene for the mITPA protein. Although we did not obtain such a sequence in the RT-PCR products from the CCE ES cells with primer set I, it is possible that the genomic sequence is transcribed in other types of cells or tissues. To clarify this point, we amplified cDNA prepared from various adult mouse tissues using primer set I, and the RT-PCR products were digested with two different restriction enzymes, *Apa*LI and *Hae* II. The former digests the processed gene-like sequence but not the three types of cDNA, while the latter digests only the

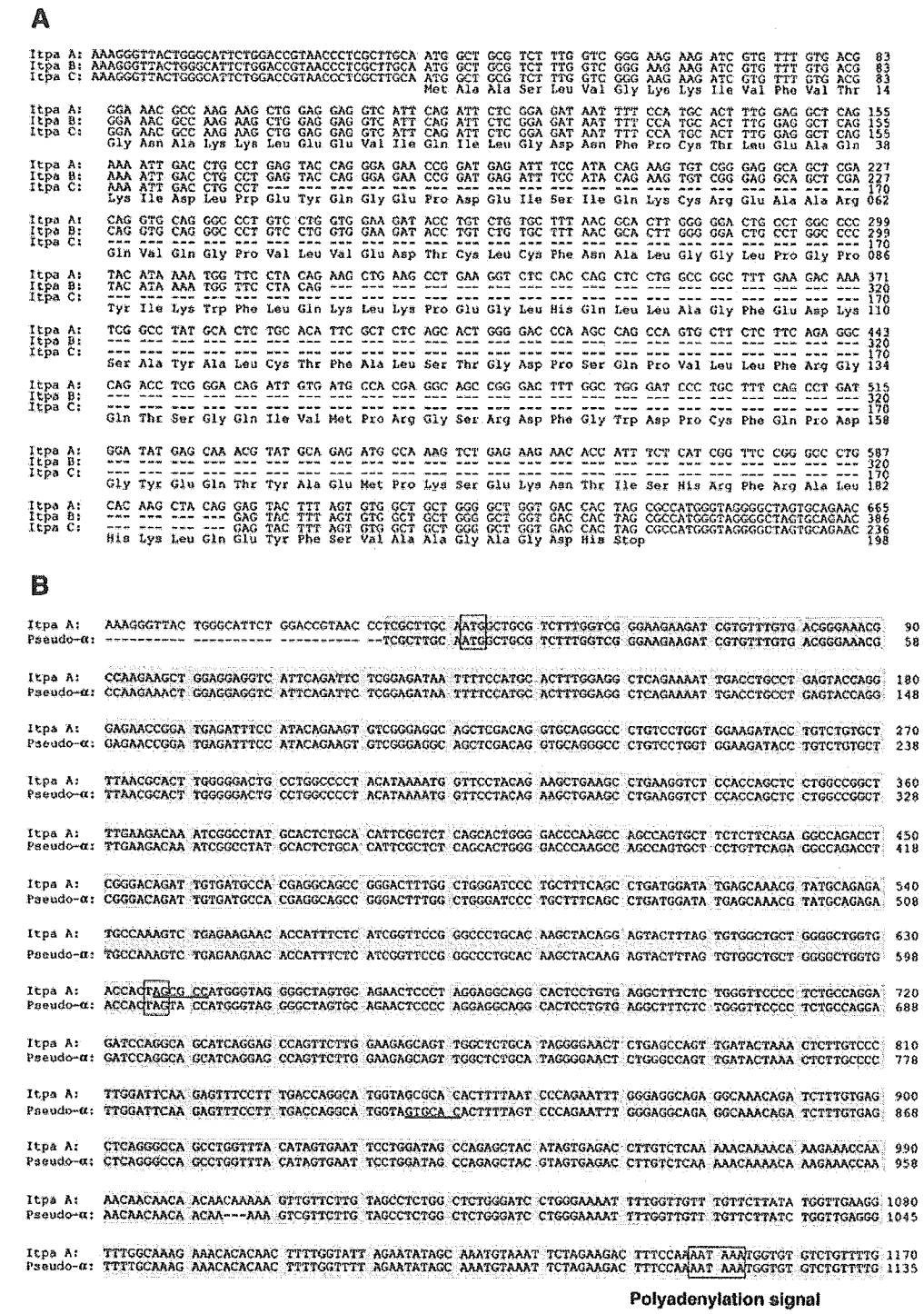



**Figure 2.** Mouse *Itpa* cDNA-related sequences. *A*. Alignment of the coding sequences of three types of mouse *Itpa* cDNA and amino acid sequences of their translation products (accession no. AB100501). The number of nucleotides and amino acid residues are shown on the right. *B*. Alignment of DNA sequences for type A *Itpa* cDNA and the genomic PCR product containing *Itpa* cDNA-like sequence. Type A *Itpa* cDNA was amplified from CCE RNA with a primer set of 5-3 mIT and 3-4 mIT, and the genomic PCR product was obtained from CCE genomic DNA with the primer set of Pα-45 and Pα-46 (Table 1) designed based on a mouse genome sequence (accession no. AL672251) which contained a sequence identical to that of the genomic PCR product shown in Fig. 1B (lane 2). Identical nucleotides between the two sequences are shaded and the initiation and stop codons and the polyadenylation signal are boxed. The recognition sequences for *Hae* II in the *Itpa* cDNA and *Apa*LI in the genomic PCR product are underlined.

three types of cDNA for mITPA (Fig. 2B, 3A). A single 1108-bp fragment was amplified from CCE genomic DNA pre-treated with DNase-free RNase A, while two or three bands corresponding to 1111-, 832-, or 688-bp fragments were detected in the RT-PCR products from all the tissues examined as well as from the CCE cells (Fig. 3B).

*Apa*LI digestion of the 1108-bp genomic PCR product yielded 814- and 294-bp fragments as predicted from its sequence (Fig. 3C, lane 10), while no such digested band was detected in the RT-PCR products from all adult mouse tissues examined as well as from the CCE ES cells (Fig. 3C, lanes 2–9). On the other hand, *Hae* II digestion produced 609- and 502-bp fragments from all RT-PCR products (Fig. 3D, lanes 2–9), and the genomic PCR product remained undigested (Fig. 3D, lane 10). From the cDNA prepared from the lung, cerebrum and cerebellum as well as from CCE cells, three fragments — 1111-bp, 832-bp, and, to a much lesser extent, 688-bp fragments — were amplified, and the latter two fragments were also digested by *Hae* II but not by *Apa*LI. These results were confirmed by simultaneous digestion with the two enzymes (Fig. 3E).

As a result, we concluded that the 1108-bp genomic sequence highly homologous to the mouse *Itpa* cDNA sequence is not transcribed in any of the adult mouse tissues examined so far, and thus we designated the genomic sequence as *Itpa* processed pseudogene $\alpha$ (accession no. AB100502). Furthermore, we confirmed that the three types of *Itpa* transcripts identified in CCE ES cells were also expressed in adult mouse tissue.

### 3.3. *Type A* Itpa *mRNA encodes a functional deoxyinosine triphosphate pyrophosphatase*

In order to confirm that a polypeptide encoded by the type A *Itpa* transcript possesses the capability of hydrolyzing nucleotides such as ITP or dITP, the type A cDNA was placed under the control of T7 promoter in pET8c or pET32a. In *E. coli* cells harboring pET8c:mITPA or pET32a:mITPA plasmids, a single 22-kDa or 34-kDa polypeptide was expressed after IPTG induction, respectively (Fig. 4A, lanes 5, 11). The former corresponds to the native mITPA protein with the expected molecular weight of 21,883 Da, and the latter is a fusion protein with thioredoxin (Trx-mITPA) whose molecular weight is predicted to be 33,558 Da. Crude extracts prepared from *E. coli* cells harboring vector itself or pET8c:mITPA, pET32a:mITPA were incubated with dITP or dATP, and the reaction products were separated on a DEAE column attached to an HPLC system.[12] Extracts from *E. coli* cells harboring vector itself had no detectable activity to hydrolyze dITP (Fig. 4B, top panel; Fig. 4C, open circles and squares), while the extracts from *E. coli* cells expressing mITPA efficiently converted dITP to dIMP (Fig. 4B, bottom panel; Fig. 4C,

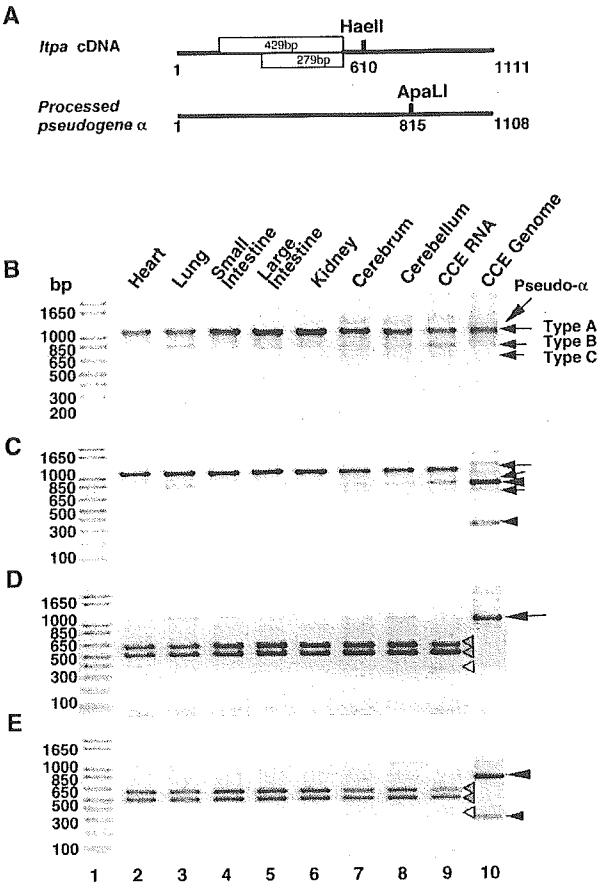
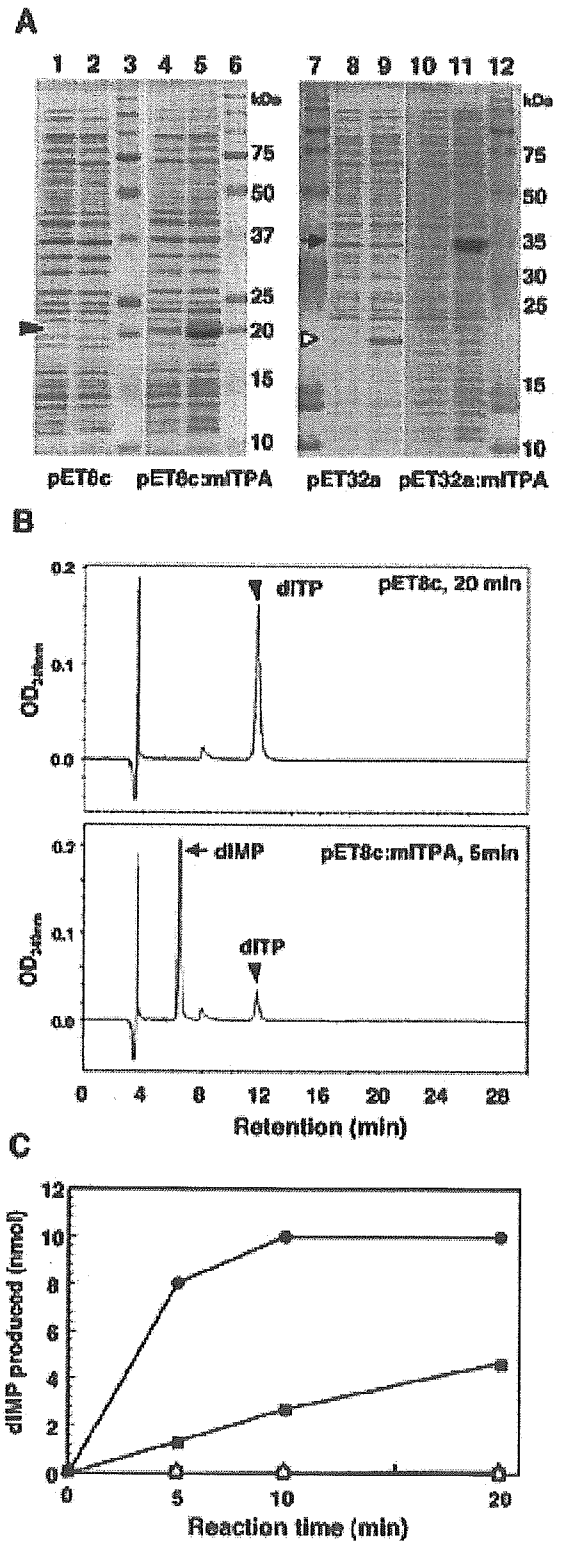



**Figure 3.** Transcripts for mouse ITPA expressed in adult mouse tissue. *A.* Schematic representation of the DNA sequences for type A *Itpa* cDNA (top) and the genomic PCR product containing *Itpa* cDNA-like sequence (bottom). The recognition site for *Hae* II or *Apa*LI in each sequence is shown, and sequences missing in type B and C *Itpa* cDNA are shown with open boxes. *B.* Agarose gel electrophoresis of the RT-PCR products from adult mouse tissue. cDNAs reverse-transcribed from total RNA prepared from C57BL/6J adult mouse tissue specimens (lanes 2–8) and CCE cells (lane 9), were used as templates for RT-PCR with primer set I shown in Fig. 1A. RNAs from lung, heart and kidney as well as CCE cells were pre-treated with RNase-free DNase I before cDNA synthesis in order to avoid any contamination of genomic DNA. Genomic DNA from CCE cells was treated with DNase-free RNase A (lane 10). *C.* Digestion of the PCR products with *Apa*LI. *D.* Digestion of the PCR products with *Hae* II. *E.* Digestion of the PCR products with *Apa*LI and *Hae* II. The longest arrow indicates the genomic PCR fragment of 1108 bp. RT-PCR products for type A (1111 bp), B (832 bp), and C (688 bp) are also shown with three different sizes of arrows. Two large and small closed arrowheads indicate the digested bands (814, 294 bp) derived from the genomic PCR fragment. Gray arrowheads indicate *Hae* II-digested RT-PCR fragments (609, 502 bp) derived from type A *Itpa* cDNA, open arrowheads indicate the *Hae* II-digested RT-PCR fragment (330 bp) derived from type B *Itpa* cDNA.




closed circles). Extracts containing Trx-mITPA also hydrolyzed dITP to dIMP (Fig. 4C, closed squares). dATP was barely hydrolyzed by extracts prepared from *E. coli* cells with or without mITPA (Fig. 4C, triangles), thus demonstrating that mITPA encoded by the type A *Itpa* mRNA specifically hydrolyzes dITP into dIMP.

### *3.4. Isolation and characterization of mouse* Itpa *gene*

A detailed analysis of mouse genomic sequences revealed that there are several pseudogene-like sequences highly homologous to mouse *Itpa* cDNA in the mouse genome (Table 2). In order to isolate the functional mouse *Itpa* gene, we initially intended to isolate unique intronic sequences for mouse *Itpa* gene. Based on the genomic structure of the human *ITPA* gene consisting of 8 exons and 7 introns,[24,25] each exon of the mouse *Itpa* gene was predicted based on the type A mouse *Itpa* cDNA sequence, and each intron sequence, except for introns 1 and 6, was amplified from genomic DNA prepared from CCE ES cells by two primers hybridized to the adjacent exons (Table 1).

With the genomic fragment containing the intron 2 or 3 as a probe, we applied the retro-recombination method,[29] in order to isolate the genomic sequences encompassing *Itpa* gene from a λTK phage genomic library derived from

**Figure 4.** Type A *Itpa* mRNA encodes functional deoxyinosine triphosphate pyrophosphatase. *A*. The expression of recombinant mITPA proteins in *E. coli* cells. Isopropyl β-D-thiogalactoside (IPTG, 1 mM) was added to the exponentially growing cultures of *E. coli* BL 21 cells carrying pET8c (lanes 1, 2), pET8c:mITPA (lanes 4, 5) or pET32a (lanes 8, 9, pET32a:mITPA (lanes 10, 11), and each culture was further incubated at 37°C for 3 hr. An aliquot was harvested before (lanes 1, 4, 8, 10) and after the addition of IPTG (lanes 2, 5, 9, 11). Whole cell extracts prepared from each cell were subjected to 15% (Lanes 1–6) or 12.5% SDS-PAGE (lanes 7–12), and the gels were stained with Coomassie brilliant blue. Lanes 3, 6, 7, 12, molecular weight markers. The closed arrowhead indicates a band corresponding to the 22-kDa mITPA protein, an arrow indicates the 34-kDa Trx-mITPA fusion protein, and the open arrowhead indicates the Trx itself (20 kDa). *B*. Hydrolysis of dITP by the recombinant mITPA. dITP (1 mM) was incubated in reaction mixture (10 μl) contained 50 mM Tris-HCl, (pH 8.5), 50 mM $MgCl_2$, 1 mM DTT, with 50 ng of whole cell extracts prepared from IPTG-induced *E. coli* cells carrying pET8c for 20 min (top panel) or pET8c:mITPA for 5 min (bottom panel), at 30°C. The reaction was terminated by adding 5 mM EDTA, then the products were separated on a TSK-Gel DEAE-2SW column, as previously described.[12] *C*. mITPA specifically hydrolyzes dITP but not dATP. Whole cell extracts (50 ng of protein) were incubated with 1 mM dITP or dATP for the time noted, and the products were separated on a TSK-Gel DEAE-2SW column. The amount of nucleotides was determined by UV absorbance at 249 nm. Closed circles, dITP reacted with extracts prepared from cell carrying pET8c:mITPA; open circles, dITP reacted with extracts prepared from cells carrying pET8c; closed squares, dITP reacted with extracts prepared from cells carrying pET32a:mITPA; open squares, dITP reacted with extracts prepared from cells carrying pET32a; closed triangles, dATP reacted with extracts prepared from cells carrying pET8c:mITPA; open triangles, dATP reacted with extracts prepared from cell carrying pET8c.

**Table 2.** Restriction fragments containing a homologous sequence with mouse type A *Itpa* cDNA predicted from the mouse genome database.

| Nomenclature | Chromosome | DDBJ/EMBL/GenBank accession no. | Restriction fragment size (kbp) *Eco*RI | *Hin*dIII | *Xba*I |
|---|---|---|---|---|---|
| *Itpa* gene | 2F3 | AL772162<br>AB101662 | 3.17, 7.4, 9.95 | 0.84, 0.91, 2.35, 21 | 4.7, 10.4 |
| *Itpa* pseudogene α | 2E1 | AL672251<br>AB100502 | 5.9, 12.7 | 4.8 | 4.7, 7.4 |
| *Itpa* pseudogene β | 9E4 | AC091531 | 11.8, >2.6 | 9.8 | 12.5 |
| *Itpa* pseudogene γ | XA3.2 | AL672147 | 8.8 | 4 | 4.4 |

129 SvJ mouse. Fourteen clones were isolated and classified into three groups as shown in Fig. 5A. The sequences of PCR products and λTK phage clones together with the mouse DNA sequence from clone RP23-175J8 on chromosome 2 (accession no. AL772162) revealed that mouse *Itpa* gene consists of 8 exons and 7 introns as expected from the genomic structure of the human *ITPA* gene, and it spans about 13.8 kb of the region on the F3 band of chromosome 2 (Table 2, Fig. 5A,B). The alignments of type B and C *Itpa* cDNA sequences with the genomic sequence revealed that the two transcripts are generated by an unusual joining between exon 5 and exon 8 or between exon 3 and exon 8, respectively (Fig. 5A).

Type A mRNA, a major transcript from *Itpa* gene, is generated by splicing at canonical splice sites with GT-AG dinucleotides at the splice junctions.[32,33] However, type B and C transcripts are most likely generated by splicing at non-canonical splices sites with GA-AG or AA-AG dinucleotides at each unusual splice junction, respectively (Fig. 5B).[33] In both cases, the same 3′-acceptor site in the exon 8 was used. The 5′-donor sequence (*GAGTAC*) in exon 3 was also found at the 5′-end of alternatively spliced exon 8, while the sequence (*CTACAG*) at the 3′-end of alternatively spliced exon 5 was also present at the 3′-acceptor site in exon 8, thus suggesting that these repeated sequences may be involved in such unusual splicing.

To confirm the genomic structure for the mouse *Itpa* gene, genomic DNA prepared from ES cells was digested with restriction enzymes *Eco*RI, *Hin*dIII or *Xba*I, and subjected to Southern blot hybridization with a DNA fragment containing the entire coding region from type A cDNA (731 bp) as a probe (Fig. 6). In addition to the bands expected from the genomic sequence for *Itpa* (Table 2; Fig. 6, arrows), three extra bands for each digested sample were identified which likely represent the processed pseudogenes α, β, and γ, respectively (Fig. 6, arrowheads). Among them, the *Itpa* pseudogene α located on chromosome 2E1 has a complete ORF for exactly the same polypeptide as ITPA encoded by the type A transcript, with a polyadenylation signal (Fig. 2B). However, we could not detect any transcribed sequence derived from the *Itpa* pseudogene α in CCE ES cells or from any mouse tissues examined, as shown in Fig. 3, thus concluding that the mouse has only one functional *Itpa* gene in its genome.

The existence of such processed pseudogenes in the mice were confirmed by Southern blot analysis of 129, C57BL/6J and BALB/c mouse strains, and essentially the same results were obtained from all strains examined as well as for CCE ES cells (data not shown). Although there was one more pseudogene-like sequence on chromosome 6 in the mouse genome database, we could not identify any band corresponding to the sequence in our Southern blots (Fig. 6). This may be because the sequence has much less homology with the *Itpa* cDNA probe used.

### *3.5. Expression of mouse* Itpa *gene in mouse tissues and its association with cell proliferation*

Expression of *Itpa* gene in adult mouse tissues was examined by a Northern blot analysis (Fig. 7A, top panel). A band corresponding to 1.35 kb in length was detected in all examined tissues, however, its levels significantly varied from tissue to tissue. Measurements of the radioactivities of bands on the blot revealed the highest expression to be seen in the testis and brain, followed by the thymus (Fig. 7A, lanes 2, 13, 14), and most other tissues except for smooth muscle, the salivary gland and stomach, expressed about a 50% of the level of *Itpa* mRNA detected in the brain or testis (Fig. 7A, bottom panel).

We then monitored the expression of *Itpa* in serum-starved BALB/c 3T3 cells with or without serum stimulation (Fig. 7B). A quantitative analysis of the Northern blots revealed the level of *Itpa* mRNA in quiescent cells to be low, however, this level increased twofold within 15 hr after serum stimulation and then gradually decreased after entering the S phase, 18 hr after serum stimulation, and then returned to the basal level after 24 hr or later (Fig. 7C).

The genomic sequence of the *Itpa* gene revealed a

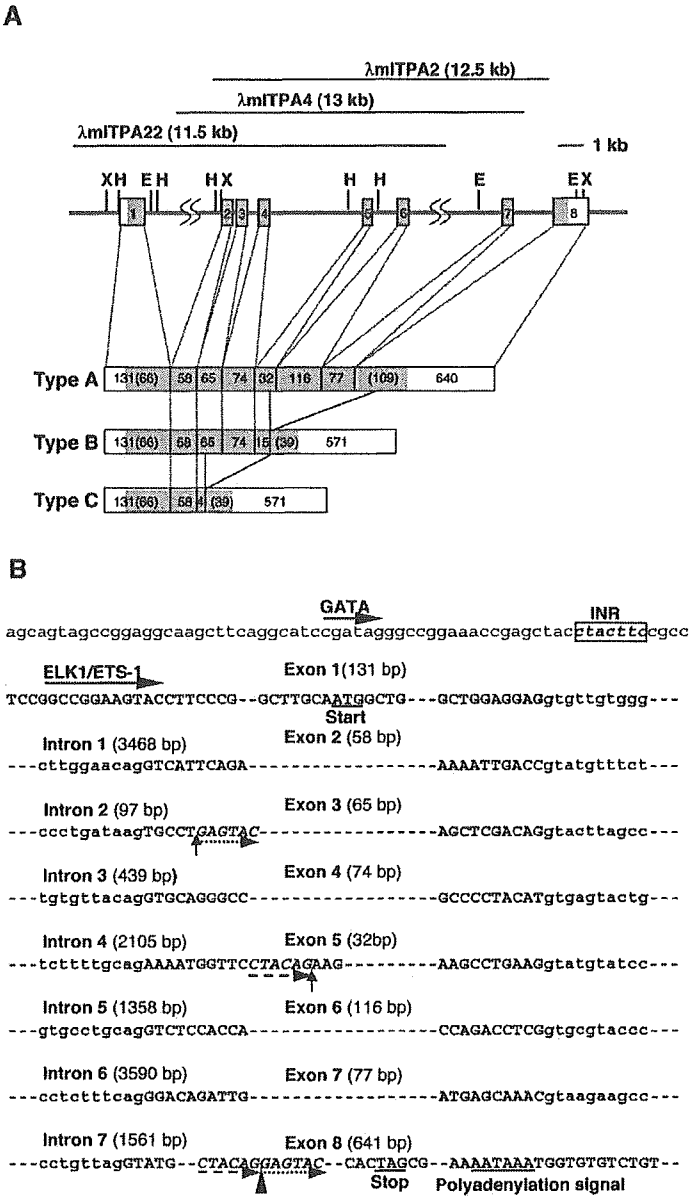



**Figure 5.** Genomic organization and alternative splicing of the mouse *Itpa* gene. *A*. A schematic diagram of the structure of the *Itpa* gene and its transcripts. The alignment of the DNA fragments derived from mouse genomic libraries is shown in the upper part. The approximate insertion size of each clone is also shown in parentheses. In the middle part, the structure of the gene, together with the appropriate restriction enzyme sites (E: *Eco*RI, H: *Hin*dIII, X: *Xba*I), is shown. The boxes represent the exons for the *Itpa* gene and the shaded regions represent the protein-coding region. In the bottom part, three types of alternatively spliced *Itpa* mRNAs are shown. The sequence derived from each exon is shown as a box with a number of bases consisting of each exon, and the dashed lines indicate the normal splicing sites, while solid lines indicate the unusual splicing sites found in type B and type C transcripts. A number of bases for the protein-coding region derived from exon 1 or exon 8 are shown in parenthesis. *B*. The nucleotide sequences of intron/exon boundaries of *Itpa* gene. The nucleotide sequences of the exons and parts of the introns determined by a comparison of the sequence of type A *Itpa* cDNA with the mouse genomic sequence (Accession nos. AL772162, AB101662), are shown in bold uppercase and lowercase, respectively, and the flanking sequences in plain lowercase. The genomic sequences shown were confirmed by sequencing of phage clones from a λTK phage genomic DNA library derived from 129SvJ mouse or genomic PCR products. The start of exon 1 was based on the most 5′-extended EST clone for *Itpa* mRNA (Accession no. BB654208). The initiation codon ATG, the termination codon TGA and a putative polyadenylation signal are underlined. The arrows indicate the unusual 5′-splicing sites in the exon 3 and 5, and the arrowhead indicates the unusual 3′-splicing site in the exon 8, found in type B and type C transcripts. The dotted lines indicate the repeated sequences at the unusual splicing junctions.