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Intron 1 and the 5′-flanking region of the human thymidylate synthase gene as a regulatory determinant of growth-dependent expression

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ABSTRACT

We have determined the regulatory regions responsible for the growth-dependent expression of the human thymidylate synthase (TS) gene, using a set of minigenes constructed from segments of the human TS gene and the cDNA clone. Each construct was introduced stably into a TS-negative mutant of rat fibroblast 3Y1 cells. By serum-restricted synchronization of the cloned transformant cells, we found that a minigene with the genomic 5′-flanking region and intron 1 without other introns were sufficient for the normal extent and pattern of S-phase specific expression at the levels of both mRNA and enzymatic activity. In contrast, a TS cDNA clone driven by an SV40-based expression vector showed constitutive expression. Insertion of intron 1 into the cDNA clone in the normal location, or replacement of the viral 5′-promoter region of the cDNA clone by the genomic 5′-flanking sequence converted the constitutive expression to the S-phase dependent one, but only partly, that is, coexistence of the two regions were required for the normal expression. Results obtained by nuclear run-on assay suggested that posttranscriptional controls are also involved in this regulation in consistent with our previous results with the *bona fide* human TS gene.

INTRODUCTION

Thymidylate synthase (TS, EC 2.1.1.45) catalyzes the only pathway for the de novo synthesis of dTMP, and plays a key role in balancing the four nucleotide precursors for DNA replication. Impairment of this enzyme causes various biological and genetic abnormalities (1,2)

We have isolated genomic DNA clones covering the entire human TS gene and its cDNA clone, and determined their complete nucleotide sequences (3–5). The 5′ upstream regions of the human and mouse genes are G+C-rich and lack canonical transcriptional signals, such as a TATA box, and CAAT box, like those of some other housekeeping genes (5,6).

The TS activity and mRNA content are associated with cell proliferation. They are almost null in non-cycling cells, but increase sharply 20- to 40-fold at the G_1-S border during serum-induced transition from the resting (G_0) phase to the S phase in human diploid fibroblasts (7) and mouse 3T6 cells (8). Similar growth-dependent increases have been observed in some other growth-associated genes involved in DNA synthesis, such as dihydrofolate reductase (9), thymidine kinase (TK) (10), DNA polymerase α (11) and proliferating cell nuclear antigen (PCNA) (12). However, nuclear run-on analysis revealed that the transcriptional activity of the TS gene in human and mouse cells increased at most only two to four times in the above transition(7,8). This suggests that posttranscriptional events must be important for accumulation of the mRNAs during G_0-S phase transition.

In this study we tried to determine which region of the human TS gene is responsible for the S-phase-dependent increase of the TS mRNA with the use of chimeric minigenes stably introduced into a TS-negative mutant of rat 3Y1 cells. Our results show that intron 1 and the 5′-flanking sequence are each independent determinants of cell cycle-directed expression, but coexistence of the two are required for the full expression equivalent to that of the *bona fide* gene.

MATERIALS AND METHODS

Enzymes and chemicals

The sources of materials used in this work were as follows: [α-^{32}P]dCTP (3000Ci/mmol,1Ci=37GBq), [α-^{32}P]UTP (400Ci/mmol) and GeneScreen membrane sheets from Du Pont-New England Nuclear, random primer DNA labeling kit from Takara Shuzo (Kyoto), [5-^{3}H]dUMP (18Ci/mmol) and aqueous counting scintillant ACS II from Amersham, ES medium from Nissui Seiyaku (Tokyo), and fetal calf serum (FCS) from Biocell Laboratory (Carson,CA).

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Cell lines and culture conditions

TS-negative rat 3Y1 fibroblastic cell mutants (thymidine auxotrophs) were isolated previously (5). One clone, TS-6, was cultured in ES medium containing 10% FCS and 20μM thymidine. Its prototrophic transformants were cultured in thymidine-free medium. For experiments, cells were cultured to subconfluency, and made quiescent by treatment with 0.5% FCS for 4 days. The medium was changed on day 2. The quiescent cells were stimulated to proliferate in ES medium with 20% FCS. After 21 hr, the rate of [^{3}H]thymidine incorporation into the acid insoluble fraction reached a maximum in TS-6 cells and their transformants (data not shown). Therefore, we used cells at this time as S-phase cells.

Construction of minigenes

A recombinant plasmid clone for the functional human TS cDNA clone (pcHTS-1) and a minigene (pmHTS-1) containing intron 1 were described by Ayusawa et al. (3) and Kaneda et al. (5), respectively. pmHTS-0 was made by substituting a 0.7kb *Sph*I-AsuII fragment of the TS coding region of pcHTS-1 for a 2.4kb *Sph*I-AsuII fragment containing intron 1 of pmHTS-1, and pcHTS-1-i1 was made *vice versa*. pmcHTS-0 is a chimeric minigene consisting of the 5'-half (4 kb, *Xho*I-*Apa*I fragment) of pmHTS-0 and the 3'-half (3.8 kb, *Apa*I-*Sal*I fragment) of pcHTS-1, and pcmHTS-0 has the reverse structure. The 5' flanking region of pmcHTS-0 is shorter than that (3.8kb) of pmHTS-0 due to deletion of a 0.4 kb *Eco*RV-*Xho*I fragment. The structures of these minigenes are shown in Fig.1A.

Transformation of rat 3Y1 TS-6 cells

The minigene and pSV2neo were cotransfected into 3Y1 TS-6 cells by the calcium phosphate coprecipitation method described elsewhere (13). Thymidine prototrophic transformants were isolated after growing the cells in ES medium containing 10% dialyzed FCS and 100μg per ml Geneticin (GIBCO).

Northern blot analysis

The quiescent cells and growth-stimulated cells were lysed on dishes in lysis solution (8M guanidine-HCl, 20mM EDTA, 1mM dithiothreitol and 10mM sodium acetate pH 5.2). Total RNA samples were prepared from the lysates and separated by electrophoresis in 1.2% agarose containing formaldehyde as described previously (7). The RNAs were blotted onto a GeneScreen membrane in a VacuGene Vacuum Blotting system (Pharmacia, Sweden) after partial alkaline hydrolysis of the RNA as described in the supplier's manual. Then RNA on the membrane was crosslinked by UV-treatment, and hybridized to a probe prepared with a random primer labeling kit with [α-^{32}P]dCTP. Hybridization, washing and rehybridization were performed according to the instruction manual of GeneScreen. Then, membranes were autoradiographed on an X-ray film backed with an intensifying screen at −80°C.

Assay of TS activity

TS activity was assayed radiochemically by measuring tritium release from [5-^{3}H]dUMP as described previously (14).

Nuclear run-on assay

The labeled nascent transcripts in the isolated nuclei were obtained as described previously (7). The following double stranded DNA probes for detection of nascent transcripts were denatured and immobilized by UV-treatment on GeneScreen: 0.12μg or 0.2μg of BglI-EcoRI fragment (BE in Fig. 1B, 178bp) and 0.54μg or 0.9μg of EcoRI-HpaI fragment (EH in Fig. 1B, 935bp), respectively, of human TS cDNA and 0.6μg or 1.0μg of the cDNA fragment (1.3kb) of human glyceraldehyde-3-phosphate dehydrogenase (15) and 0.6μg or 1.0μg of λ phage DNA. Hybridization and washing conditions were as for Northern blot analysis. Then, the membranes were washed and exposed to an imaging plate and this was analyzed with FUJIX BAS 2000 (Fuji Photo Film, Tokyo)

RESULTS

Structures and expressions of minigenes

Previously, we isolated a functional human TS cDNA clone, pcHTS-1 which was under control of SV40 early promoter and its 3'-flanking region (3). We also isolated recombinant phage clones covering the human TS gene (16). By use of these clones, we constructed a minigene pmHTS-1(having intron 1) and several

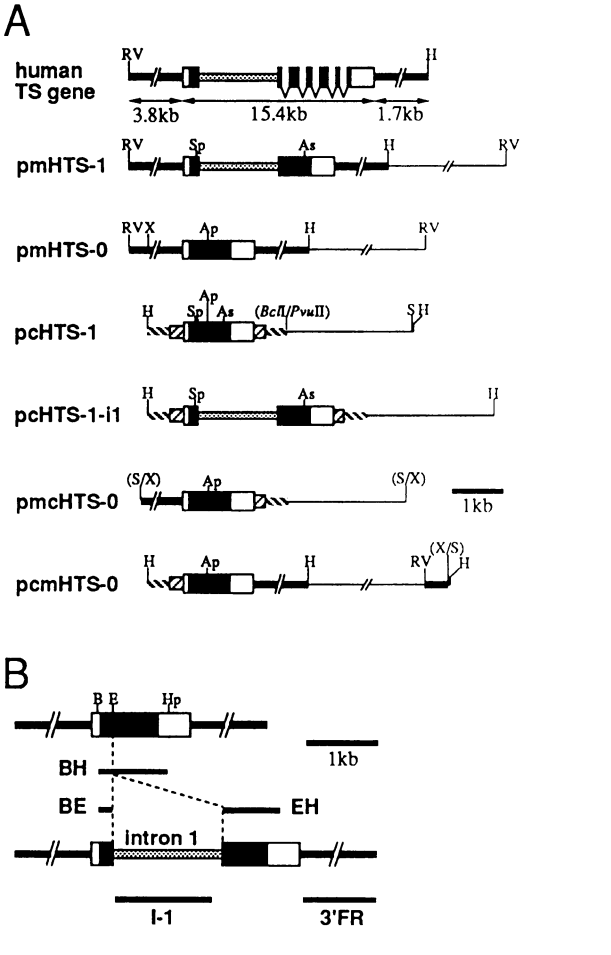

Fig.1. (A) Structures of human TS gene and minigenes. (B) The probes used for Northern blot analysis and nuclear run-on assays. Symbols: ■, TS coding region; □, untranslated region derived from TS cDNA ; ▨, untranslated region derived from SV40; ▩, intron 1(1.7kb); ▬, 5'- or 3'-flanking region of TS gene; ▥, 5'- or 3'-flanking region derived from SV40; ——, pBR322 DNA. The human TS gene and all introns except intron 1 are not drawn to scale. BE is the portion of exon 1 and EH is the portion from exon 2 to 7 of cDNA fragment BH. Abbreviations: Ap, *Apa*I; As, *Asu*II; B, *Bgl*I; E, *Eco*RI; H, *Hind*III; Hp, *Hpa*I RV, *Eco*RV; S, *Sal*I; Sp, *Sph*I; X, *Xho*I;(*Bcl*I/*Pvu*II), (S/X) and (X/S) designate restriction enzyme sites lost by ligation.

other kinds of chimeric minigenes by combination of fragments derived from pcHTS-1 and from pmHTS-1, as shown in Fig.1A. pmHTS-0 is identical with pmHTS-1 except that it does not have intron 1, and pcHTS-1-i1 is identical to pcHTS-1 except it has intron 1 in the normal location in the coding region. pmcHTS-0 is a chimeric minigene recombined in cDNA, consisting of the 5′-half of pmHTS-0 and the 3′-half of pcHTS-1, and pcmHTS-0 is *vice versa*.

In this work, we transformed rat 3Y1TS-6 cells stably by transfecting them with each of the TS minigenes. In each case at least three independent cell clones were isolated. The human TS mRNA levels in resting and growing cells were determined by Northern blot analysis(Fig. 2). As described in Materials and Methods, resting cells were cells made quiescent in low serum medium and growing cells were those stimulated to proliferate in medium with 20% FCS.

Three or more independent clones for each kind of transformant cell gave similar results, so here we show the results obtained with one clone of each type. Intensities of bands of mature TS mRNA were measured by densitometric scanning and calculated the ratio of the S phase to the G_0 phase values (data not shown). The ratios of the mRNA were similar to those of TS activity (the right column in Fig.3). However, we noticed that the variations in TS mRNA levels in clones transformed with pmHTS-0 and pcHTS-1-i1 were greater than those in clones transformed with pmHTS-1 or pcHTS-1. We did not examine the reason for this difference.

As shown in Fig. 2, pmHTS-1 functioned normally with respect to growth-dependent regulation. In this case, S-phase cells gave a strong band of mature mRNA (faster migrating band) as compared to resting cells where the mature mRNA band is scarcely seen. In contrast, the level of TS mRNA driven by pcHTS-1 seemed constant on per cell basis throughout the G_0-S transition, although the apparent mRNA level of pcHTS-1 in resting cells was about twice that in growing cells. This is explained by the fact that the total RNA content per cell doubles during traverse from the resting state to the S phase, and a constant amount of total RNA was applied to each lane of the gel. The equal amount of loaded RNA in each lane(see legend to Fig. 2) were confirmed by staining the ribosomal RNAs in the gel. Slower migration of the TS mRNA by pcHTS-1 than that by pmHTS-1 can be explained by the different length in the 5′- and 3′- untranslated regions of the two transcripts. In contrast, the TS mRNA level of pmHTS-0 (identical to pmHTS-1 except having no intron) in resting cells was significantly high. The results show that this difference is due to the presence and absence of intron 1 in the two minigenes. It should be emphasized that the increase of TS mRNA in the S-phase by pmHTS-1 is the same as that of the *bona fide* TS gene in normal human diploid cells, indicating that intron 1 without other introns in the minigene can exert its normal activity. Characterization of the higher molecular weight bands seen in Fig. 2 was described in the Discussion .

As the expression of TS in the pcHTS-1-transformant cells was constitutive, the growth-dependent expression observed in the case of pmHTS-0 should be due to its 5′- and/or 3′- flanking genomic sequence in this minigene. To investigate which of the two regions is necessary for the growth-dependent expression, we prepared chimeric minigenes, pmcHTS-0 and pcmHTS-0, the former having the genomic 5′-flanking sequence and the viral 3′-flanking sequence, and the latter having the reverse sequences (Fig.1A). Fig.2 shows that the production of TS mRNA by pmcHTS-0 increased in growing cells, whereas that by pcmHTS-0 seems constitutive. Again, the evaluation was made after normalization on per cell basis. The results indicate that the 5′-flanking sequence, but not the 3′-flanking sequence is responsible for growth-dependent increase of TS mRNA in the intronless minigene pmHTS-0.

The relative increase of mRNA in the transition from G_0 to S phase was greater in the case of pmHTS-1 than in the case of pmHTS-0. Thus, intron 1 plays a regulatory role in controlling the level of TS mRNA in the cell-cycle. To examine whether intron 1 can exert such role by itself, we inserted intron 1 into pcHTS-1 (TS cDNA driven by an SV40 promoter) to see if its constitutive nature of expression would be converted to an

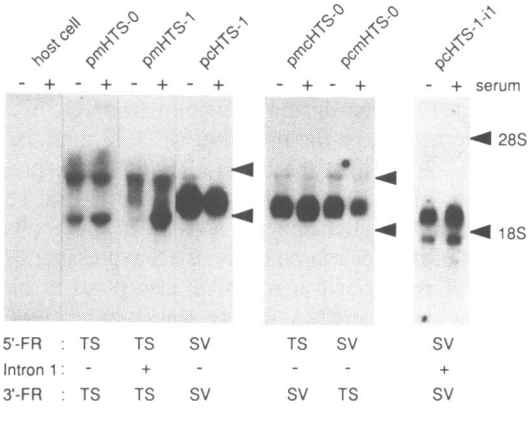

Fig.2. Northern blots of TS mRNAs derived from the minigenes. Experiments were performed as described in Materials and Methods. Total RNAs were prepared from the resting and S-phase cells (marked by minus and plus symbols for the serum condition, respectively, above the panel) and loaded on each lane of 1.2% formaldehyde/agarose gel (20μg for each lane except the rightest two lanes with 10μg). After electrophoresis, RNAs were partially hydrolyzed by alkali and transferred to a nylon membrane. BH fragment shown in Fig.1B was used as a probe. Positions of ribosomal RNAs are indicated by ◀. Composition of the minigenes are shown below the panel. 5′-FR and 3′-FR stand for the 5′- and 3′-flanking regions. TS and SV stand for the TS gene and SV40 gene, respectively, as a component of regulatory regions of minigene(see Fig. 1).

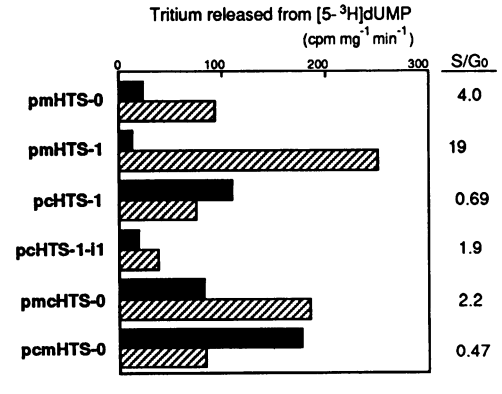

Fig.3. TS activities expressed by the minigenes. Transformants with each minigene in the resting phase (■) and S phase (▨) were sonicated and centrifuged at 100×*kg* for one hour. The supernatants were used for assay of TS activities. Assays were performed in duplicate as shown in Materials and Methods. Data in this figure were obtained with the same clones as for Fig.2. Ratios of TS activity in the S phase to those in G_0 phase were calculated and shown in the column of S/G_0.

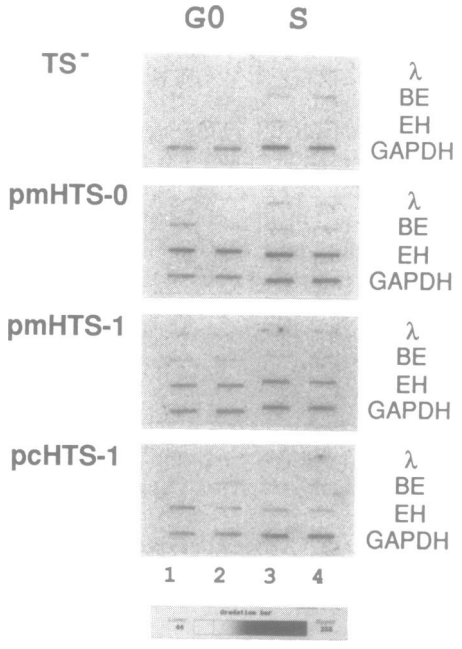

Fig. 4. Transcription of minigenes in vitro in isolated nuclei. Nuclei were isolated from resting(G_0) and S-phase cells(S) transformed with the indicated minigenes, and nuclear transcriptions were assayed as described previously(7). Data in this figure were obtained with the same clone for each minigene as used for Fig. 2. ^{32}P-labeled transcripts obtained were hybridized to a filter to which denatured DNA probe had been immobilized as described in Materials and Methods. The resulting hybridization signals were analyzed with FUJIX BAS 2000 and printed out by Pictrography(Fuji Photo Film, Tokyo). The gradation bar indicates that radioactivities of under level 44 were adjusted to zero for subtraction of the background image and a new shade gradation between level 44 and 252 was made.

inducible one. This construct was designated as pcHTS-1-i1 (Fig.1A). Interestingly, pcHTS-1-i1 acquired growth-dependent expression, but not to a full extent as shown in Fig.2. These results were reproducible with several separate experiments in terms of comparison of RNA bands between the resting cells and the S phase cells. Some of the extra minor bands seen in the case of pcHTS-1-i1 may be products of abnormal splicing between a splicing donor in the 5′-untranslated region of the SV40 sequence and the splicing acceptor at the end of intron 1 of the TS gene.

Thus, the human TS mRNA levels in both G_0 and S phases seems to be regulated cooperatively by the 5′-flanking region and intron 1 of the gene. It should also be noted that either one of these two regions alone can exert growth-dependent expression, but only in an incomplete fashion.

Human TS enzyme activities in transformant cells

We examined the TS activities in the transformants to see if there was any regulation at the translational or post-translational level. Fig.3 shows that the level of TS activity was essentially parallel with that of the TS mRNA in each case, suggesting that TS gene expression was mainly regulated before translation steps. However, among the transformants, we noticed that the mRNA level in cells with pcHTS-1 was the highest (Fig.2), but that their TS activity was not the highest. This finding could be due to incomplete sequence in the TS cDNA clone (pcHTS-1) in respect of the tandem repeats present in the 5′-untranslated sequences of the human TS mRNA (4,5).

Transcription of TS minigenes in isolated nuclei of the transformant cells

Since growth-dependent increase of human TS mRNA in normal human diploid fibroblasts has been suggested to be controlled posttranscriptionaly as shown by nuclear run-on assay (7), we examined with the same assay system whether posttranscriptional regulation also plays a role in the present minigene system. We used EH fragment which covers exons 2 to 7 but not exon 1(Fig.1B) to examine a possibility that the apparent posttranscriptional events are due to retarded elongation in intron 1 as reported for adenosine deaminase gene(27,28). As shown in Fig. 4, the intensities of the hybridization signal were essentially the same between the resting and growth-stimulated cells in each case for the three minigene. Thus, consistent with our previous observations in normal human diploid cells (7), posttranscriptional regulation is involved in the growth-dependent increase of TS mRNA in the present study.

DISCUSSION

Previously, we reported that the 20-fold increase in the TS mRNA level after growth stimulation of human diploid fibroblasts was mainly due to posttranscriptional events (7). Posttranscriptional regulation of TS has also been shown in mouse cells (8). Among the various human TS minigenes constructed, pmHTS-1 showed a similar pattern and extent of expression to the *bona fide* gene in human fibroblastic cells. The expressions of the intronless minigene pmHTS-0, a chimeric minigene pmcHTS-0 and an intron-inserted cDNA pcHTS-1-i1 were also growth-dependent, though lesser so than that of pmHTS-1. Hence, we conclude that both the 5′-flanking region and intron 1 are necessary for normal expression of the TS gene. Our conclusion is consistent with the report of Li et al. (17) that coexistence of the promoter region and a set of some introns are required for a normal level of expression of the mouse TS gene. The results obtained with pmcHTS-0 suggested that the 3′ region is not important for regulating the mRNA level or the growth-dependent expressions. In this connection, it should be noted that mouse TS mRNA lacks the 3′-untranslated region (18). Involvement of intron for proper regulation of mRNA level was also reported for human PCNA(19,20). In contrast to the TS gene, regulatory regions for expression of TK gene during transition from G_0 to S phase have been reported to be the promoter (21−23) and the coding sequence (23−25). Also, the human TK cDNA expressed by a heterologous promoter in mouse cells (23) and rat cells (26) gave rise to much higher enzyme activity in the S phase.

The results of nuclear run-on assay of TS expression suggested that the posttranscriptional mechanism also plays a role in the accumulation of the mRNA during transition from G_0 to S phase. Also, our results did not support the possibility(27, 28) that transcriptional arrest occurred in or near intron 1 of pmHTS-1 preferentially in resting cells.

What is the role of intron 1 in determining the level of human TS mRNA in the cell cycle ? The intron 1 sequence in the TS pre-mRNA could be responsible for the rapid degradation occurring specifically in resting cells, since TS mRNA levels in resting cells with intronless minigenes such as pmHTS-0 and pcHTS-1 were much higher than those with pmHTS-1.

The higher molecular weight bands detected in the Northern blots of transformants with pmHTS-0 and pmHTS-1 (Fig. 2) were characterized by rehybridizing the same filter to the probe representing intron 1 or the 3′-flanking region which is distal

to the poly(A) attachment site (see Fig.1B). The probe for intron 1 (I-1) hybridized to only the higher molecular weight bands derived from pmHTS-1, while the probe for the 3'-flanking region (3'FR) hybridized to the bands from both pmHTS-1 and pmHTS-0. However, similar migration of the higher molecular weight bands with the two minigenes were not consistent with the different length of the expected primary transcripts between the two minigenes. Therefore, these higher molecular weight bands are likely to be the primary, but somewhat abnormal transcripts. It is noted that Northern blot analysis of the poly(A)$^+$ RNA fraction did not give rise to the higher molecular weight bands. It is also mentioned that DNase I treatment did not eliminate the slower migrating bands in Fig. 2. A chase experiment in the presence of actinomycin D (10μg/ml) to interrupt transcription did not reduce the higher molecular weight bands in contrast to its reduction for mature sized mRNA bands. Therefore, it is unlikely that the bands represent active precursors of mature mRNA.

As reported here, human TS minigene pmHTS-1 was expressed in a growth-dependent manner in rat cells in the same way as the *bona fide* gene in human cells. This suggests that common mechanisms for regulating the TS gene exist in different species. This human TS minigene/rat TS-negative cell system is simple and should be useful for elucidating cis- and trans-acting elements participating in posttranscriptional regulation of the TS gene.

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REFERENCES

1. Seno,T., Ayusawa,D., Shimizu,K., Koyama,H., Takeishi,K. and Hori,T. (1985) In de Serres,F.J.(ed.), Genetic Consequences of Nucleotide Pool Imbalance, Plenum Publishing Corp., New York, pp. 241–263.
2. Ayusawa,D., Koyama,H., Shimizu,K., Kaneda,S., Takeishi,K. and Seno,T. (1986) *Mol. Cell. Biol.*, **6**, 3463–3469.
3. Ayusawa,D., Takeishi,K., Kaneda,S., Shimizu,K., Koyama,H. and Seno,T. (1984) *J. Biol. Chem.*, **259**, 14361–14364.
4. Takeishi,K., Kaneda,S., Ayusawa,D. Shimizu,K., Gotoh,O. and Seno,T. (1985) *Nucl. Acids Res.*, **13**, 2035–2043.
5. Kaneda,S., Nalbantoglu,J., Takeishi,K., Shimizu,K., Gotoh,O., Seno,T. and Ayusawa,D. (1990) *J. Biol. Chem.*, **265**, 20277–20284.
6. Deng,T., Li,D., Jenh,C.-H. and Johnson,L.F. (1986) *Mol. Cell. Biol.*, **264**, 16000–16005.
7. Ayusawa,D., Shimizu,K., Koyama,H., Kaneda,S., Takeishi,K. and Seno,T. (1986) *J. Mol. Biol.*, **190**, 559–567.
8. Jenh,C.-H., Geyer,P.K. and Johnson,L.F. (1985) *Mol. Cell. Biol.*, **5**, 2527–2532.
9. Hendrickson,S.L., Wu,J.-S.R. and Johnson,J.F. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5140–5144.
10. Knight,G.B., Gudas,J.M. and Pardee,A.B. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 8350–8354.
11. Wahl,A.F., Geis,A.M., Spain,B.H., Wong,S.W., Korn,D. and Wang,T.S.-F. (1988) *Mol. Cell. Biol.*, **8**, 5016–5025.
12. Jaskulski,D., Gatti,C., Travali,S., Calabretta,B. and Baserga,R. (1988) *J. Biol. Chem.*, **263**, 10175–10179.
13. Ayusawa,D., Shimizu,K., Koyama,H., Takeishi,K. and Seno,T. (1983) *J. Biol. Chem.*, **258**, 48–53.
14. Ayusawa,D., Koyama,H. and Seno,T. (1981) *Cancer Res.*, **41**, 1497–1501.
15. Tokunaga,K., Nakamura,Y., Sakata,K., Fujimori,K., Ohkubo,M., Sawada,K. and Sakiyama,S. (1987) *Cancer Res.*, **47**, 5616–5619.
16. Takeishi,K., Kaneda,S., Ayusawa,D., Shimizu,K., Gotoh,O. and Seno,T. (1989) *J. Biochem. (Tokyo)*, **106**, 575–583.
17. Li,Y., Li,D., Osborn,K. and Johnson,L.F. (1991) *Mol. Cell. Biol.*, **11**, 1023–1029.
18. Jenh,C.-H., Deng,T., Li,D., DeWille,J. and Johnson,L.F. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 8482–8486.
19. Ottavio,L., Chang,C.-D., Rizzo,M.-G., Travali,S., Casadevall,C. and Baserga,R. (1990) *Mol. Cell. Biol.*, **10**, 303–309.
20. Chang,C.-D., Ottavio,L., Travali,S., Lipson,K.E. and Baserga,R. (1990) *Mol. Cell. Biol.*, **10**, 3289–3296.
21. Lipson,K.E., Chen,S.-T., Koniecki,J., Ku,D.-H. and Baserga,R. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 6848–6852.
22. Roehl,H.H., and Conrad,S.E. (1990) *Mol. Cell. Biol.*, **10**, 3834–3837.
23. Travali,S., Lipson,K.E., Jaskulski,D., Lauret,E. and Baserga,R. (1988) *Mol. Cell. Biol.*, **8**, 1551–1557.
24. Lieberman,H.B., Lin,P.-F., Yeh,D.-B. and Ruddle,F.-H. (1988) *Mol. Cell. Biol.*, **8**, 5280–5291.
25. Stewart,C.J., Ito,M. and Conrad,S.E. (1987) *Mol. Cell. Biol*, **7**, 1156–1163.
26. Ito,M., and Conrad,S.E. (1990) *J. Biol. Chem.*, **265**, 6954–6960.
27. Chen,Z., Harless,M.L., Wright,D.A. and Kellems,R.E. (1990) *Mol. Cell. Biol.*, **10**, 4555–4564.
28. Chinsky,J.M., Maa,M.-C., Ramamurthy,V. and Kellems,R.E. (1989) *J. Biol. Chem.*, **264**, 14561–14565.