Eur J Cancer, Vol. 29A, No. 4, pp. 545–548, 1993.
Printed in Great Britain

0964–1947/93 $6.00 + 0.00

Differential Expression of Thymidine Kinase Gene in Two Subpopulations of a Rat Tumour Correlates with their Tumorigenic and Cell Division Potential

Gopal Pande and Ch Sudhakar

We have studied the expression of the thymidine kinase (TK) gene in two kinetically heterogenous populations of a rat tumour cell line—the Zajdela ascitic hepatoma (ZAH). We have demonstrated that the TK gene is differentially expressed in the two cell types. The more tumorigenic and rapidly dividing subpopulation shows higher levels of mRNA and enzyme activity for TK. In addition, we have shown that the tumorigenic cells accumulate the primary unspliced transcript and utilise only part of it for maturation. It is, therefore, likely that ZAH cells regulate their division and possibly tumorigenic potential by regulating the expression of the TK gene.
Eur J Cancer, Vol. 29A, No. 4, pp. 545–548, 1993.

INTRODUCTION

IN CONTRAST to our understanding of cell division in normal cells regulation of cell division in cancer cells is less well understood. Dysregulation in the cancer cells appears to arise due to several factors which include the actions of oncogenes, growth factors, hormones and intracellular cell cycle-specific enzymes [1–4]. One of the most critical steps in the regulation of the cell cycle is the G_1 to S transition which, among other markers, is characterised by the expression and activation of enzymes of the DNA synthesis pathway, e.g. thymidine kinase (TK) [5], dihydrofolate reductase (DHFR) [6], thymidylate synthase (TS) [7], etc. Although expression of these enzymes has been extensively studied in normal cells their significance in the tumour cells is not clear. The large variability in division rates of tumour cells along with scarcity of good model systems has made these studies even more difficult.

In an earlier report [8] we proposed that Zajdela ascitic hepatoma (ZAH), an ascitic liver tumour of the rat, can be used as a model system for studying cell cycle regulation in tumour cells. The ZAH tumour is composed of two subpopulations of cells—the heavy (H) cells and the light (L) cells, so referred because of differences in their centrifugal buoyancy densities. The H cells divide rapidly and are lethally tumorigenic, while the L cells are slow growing and induce only regressive tumours. We also showed, using fluorescence activated cell sorter (FACS) analysis of the DNA of these cells, that at a given time the H cells have about 30% cells in G_2+M phase while L cells have less than 5% cells in this phase. In the present study we have compared the expression of the TK gene and enzyme activities in the L and H cells of ZAH.

EXPERIMENTAL PROCEDURES

Cell separation

Separation of L and H cells was performed on continuous gradients of Percoll (Pharmacia, Sweden) as described earlier [8]. After separation, cells were immediately washed with cold phosphate buffered saline (PBS).

RNA dot blot and northern blot analysis

Total cytoplasmic RNA was prepared from normal liver cells, ZAH cells, L and H cells according to the procedure of Chomczynski and Sacchi [9]. Total RNA was subjected to oligo-dT-cellulose chromatography for preparing poly A^+ RNA [10].

For dot-blots, poly A^+ RNA was first denatured with 7.5% formaldehyde at 60°C for 15 min and then serially diluted in 6 × standard saline citrate (SSC). 50 μl of each sample was spotted on nitrocellulose paper (containing 2.0, 1.0, 0.5 and 0.25 μg RNA, respectively) which had been previously equilibrated with 20 × SSC. The paper was then dried and baked at 80°C for 2 h in a vacuum oven.

For northern blot experiments, total RNA samples from L cells, H cells, total ZAH cells and liver cells were run on 0.8% agarose gels in the presence of 10% formaldehyde. RNA was then blotted onto nitrocellulose paper and baked at 80°C for 2 h.

Hybridisation of both the RNA dot-blot and northern blot filters was carried out in the presence of 6 × SSC, 80% formamide and 1 × 10^7 cpm of a nick translated pHSV106 TK probe [11], for 16 h at 42°C. After hybridisation, filters were washed twice in 2 × SSC at 42°C and once in 1 × SSC at room temperature. Filters were then dried and autoradiographed at −70°C.

Southern blot analysis

DNA from all the four cell types was isolated and digested with EcoRI and PstI enzymes using standard procedures. Blotting of the digested DNA and hybridisation with the pHSV106 probe was done according to the procedures described [10].

Estimation of TK enzyme

Estimation of TK activity was done essentially according to the procedure of Lee and Cheng [12]. Briefly, cell suspensions were lysed by sonication in a buffer containing 0.01 mol/l Tris–HCl (pH 7.5), 10% glycerol, 0.15 mol/l NaCl, 20 mmol/l deoxythymidine (TdR) and 2 mmol/l dithiothreitol (DTT); all cell debris, after sonication, was spun down at 16 000 rpm for 10 min and the clear supernatant was used for further studies.

Correspondence to G. Pande.
G. Pande and C. Sudhakar are at the Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India.
Revised 15 June 1992; accepted 16 Oct. 1992.

Supernatants from the different cell types were assayed for TK activity in the following mixture: 0.19 mol/l Tris–HCl (pH 7.5), 1.9 mmol/l ATP, 1.9 mmol/l $MgCl_2$, 1% bovine serum albumin, 3 mmol/l phosphocreatine, 0.54 units creatine kinase, 10 mmol/l DTT, 10 mmol/l NaI, and 1 μCi [^{3}H]TdR (2–6 Ci/mmol). Supernatants (from equivalent of 1×10^6 cells) of each type of cell lysate were added to the above mixture as an enzyme source in a final volume of 0.1 ml each.

The assay mixture was incubated at 37°C for 30 min and the reaction was stopped by spotting 50 μl of the reaction mixture onto DE81 glass-fibre filter paper (Whatman, U.S.A.) and followed by immediate immersion in ethanol (10 ml/square). The filters were washed three times with alcohol and dried under a lamp. Radioactivity was measured by liquid scintillation counting in a Packard Tri-Carb 1500 liquid scintillation counter. Activity of the enzyme was recorded as the total adsorbed cpm as a function of the reaction time.

RESULTS

TK mRNA expression

In both dot blot and northern blot experiments, it can be clearly seen that TK-specific RNA is expressed at much higher levels in the H cells of the ZAH as compared to the L cells (Figs 1, 2). The densitometric scan of the autoradiograph showed that the levels of TK mRNA in H cells were about 10-fold higher

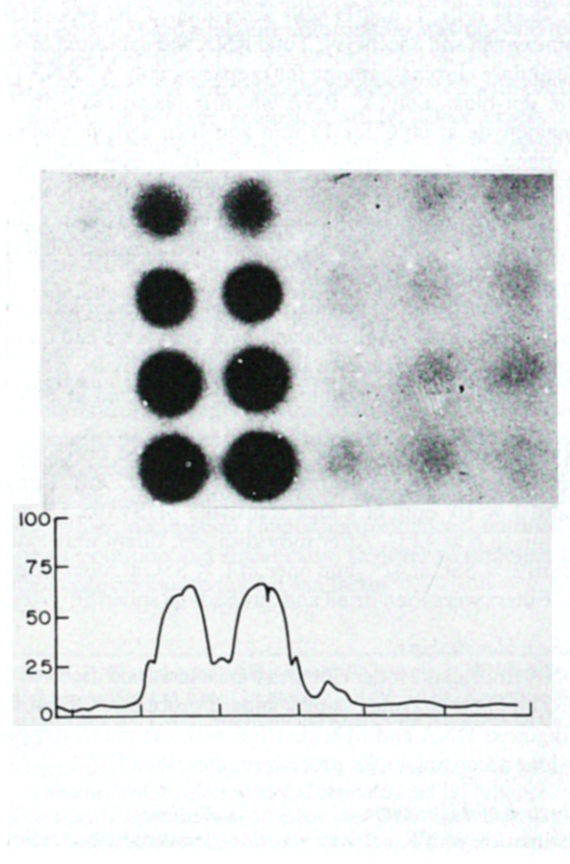

Fig. 1. RNA dot-blot hybridisation of the poly A$^+$ RNA from H cells (a), total ZAH cells (b), L cells (c), liver cells (d) and regenerating liver cells (e) with ^{32}P-labelled pHSV106 probe [11]. The lower panel depicts a densitometric scan of the autoradiogram of the 2.0 μg lane, using a Zeineh Soft Laser ScannerBiomed Instruments Model SD-2DUV.

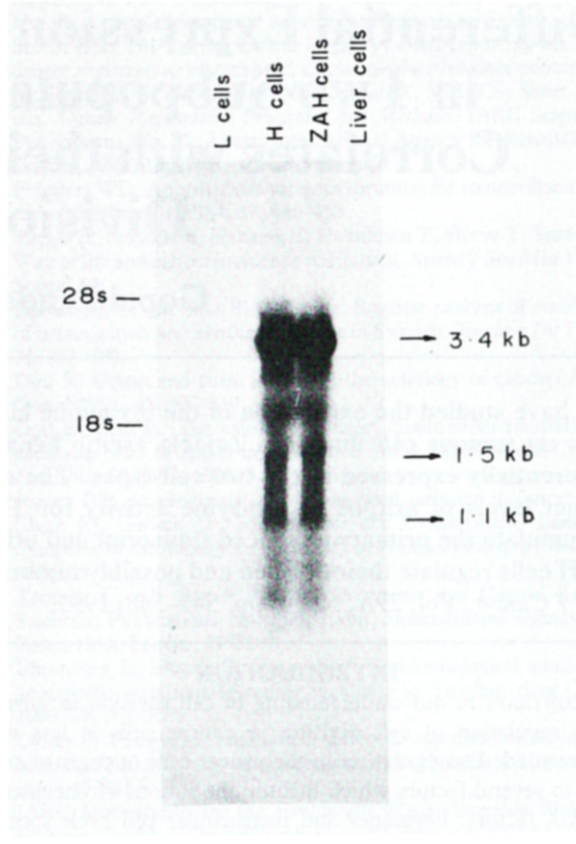

Fig. 2. Northern blot of total RNA samples hybridised to the TK probe [11]. 28S and 18S RNA positions were marked from parallel lanes of each RNA sample.

than in the L cells. Surprisingly, regenerating liver mRNA did not show very high levels of TK mRNA as compared to resting adult liver cells suggesting that the rate of turn-over of the enzyme is more in the tumour cells than when the liver cells are regenerating.

It has been demonstrated earlier that mature TK mRNA can be of heterogenous lengths ranging from 1.1 to 1.5 kb [13–15]. In our experiments three bands were detected: one major band at 3.4 kb and two minor bands at approximately 1.5 and 1.1 kb.

TK gene organisation

Southern blot analysis was performed on the DNA from all the four cell types in order to determine if there are any changes in the organisation of the TK gene at the DNA level which could alter its expression. Using two restriction enzymes, PstI and EcoRI (Fig. 3), no differences in the restriction pattern or gene dosage could be detected, indicating that the gene organisation in the two cell types is very similar if not identical.

TK enzyme activity in L and H cells

Enzyme activity of TK was measured by the method of Lee and Cheng [12]. The results have been shown in Fig. 4. We can clearly see a rapid increase in formation of [^{3}H]thymidine monophosphate both in the case of H cells and total ZAH cells indicating that the enzyme is active in the tumour cells. The L cells and liver cells show a lower level of activity. It is significant that radioactive incorporation in tumour cells declined considerably after 60 mins; this could be due to the activation of some

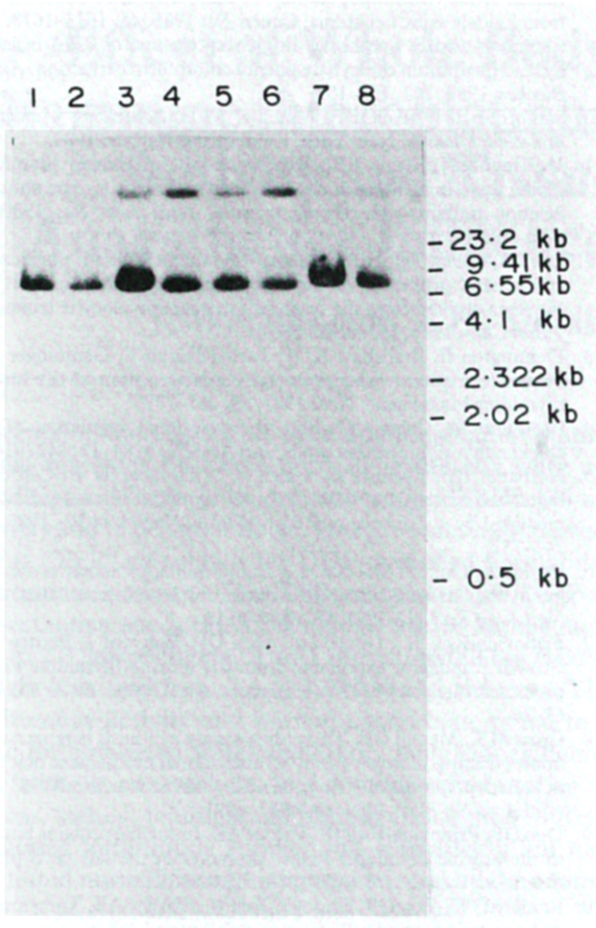

Fig. 3. Southern blot of DNA from liver (lanes 1, 2), ZAH cells (lanes 3, 4), L cells (lanes 5, 6) and H cells (lanes 7, 8) were probed with HSV-TK as described. In lanes 1, 3, 5 and 7 DNA was cut with EcoR1 and in lanes 2, 4, 6 and 8 with Pst1.

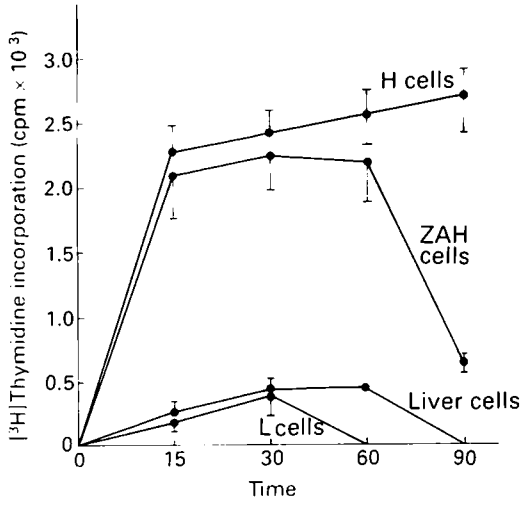

Fig. 4. Activity of the TK enzyme in cell-free extracts from different cell types, measured by the procedure of Lee and Cheng [12]. Each point in the graph represents the mean values from three independent results. The bars depict standard deviation.

non-specific phosphatases or nucleotide phosphorylases present in the total cell extracts tested.

Thus, our results show that the rapidly dividing and lethally tumorigenic subpopulation of the ZAH tumour has elevated levels of TK mRNA and enzyme activity as compared to the slow growing and regressively tumorigenic subpopulation. There are no detectable differences in the organisation of the gene between the cell types and disparate regulation of gene expression would appear to be manifested at either the transcriptional or post-transcriptional level. The presence of a major band for TK mRNA (3.4 kb) in the H cells and ZAH cells (Fig. 2) indicates that tumour cells accumulate an unspliced message in large amounts and may utilise only a part of it for making mature transcripts which are seen as minor bands.

DISCUSSION

Regulation of the TK gene is complex and occurs at the transcriptional, post-transcriptional and translational levels [16–18]. The primary mode of regulation varies between cell types, however, transcriptional control mechanisms predominate in the activity of the gene in cycling vs. quiescent cells [1]. Three specific regions between the −174 and −4 bp of the TK translational initiation site have been identified where specific transcriptional factors like Sp1, "Yi" and "Bing" are reported to bind and regulate the activity of the TK gene and other heterologous downstream genes, in an S-phase-specific manner [19]. Most of the studies on the regulation of the TK gene have been done using normal cells and it is known that cancer cells regulate their genes at the G_1–S boundary differently to normal cells [20], studies on the regulation of the TK gene in tumour cells could, therefore, be used to obtain an indirect understanding of how their proliferation is differentially regulated.

With this aim we have studied the TK mRNA levels in two kinetically heterogenous subpopulations of a rat hepatoma. The slow growing subpopulation of tumour cells (L cells), which has less than 5% cells in division phase, exhibited very low amounts of TK mRNA while the fast growing tumour cells (H cells) showed 10-fold higher levels. In the present study we have not studied the levels of the specific transcription factors of the TK gene, which have been reported by others [19], and are most likely to be responsible for this differential activity of the TK gene in the two subpopulations reported here. We have, however, shown previously that L cells are generated during the proliferation of H cells and represent a more differentiated and hence more quiescent stage of the tumour [8]. We, therefore, believe that the blockage of transcription of the TK gene occurs as a consequence of tumour progression, i.e. when a small percentage of the rapidly dividing H cells differentiate and become postmitotic and regressively tumorigenic. This kind of phenomenon has been described for specific genes in other tumours [21].

Another important point noticed in our experiments was the size of the TK transcript. We see a major band of about 3.4 kb, which is much larger than that reported earlier for murine TK. The normal size of mature TK mRNA may vary from 1.5 to 1.1 kb coding for a protein of 42 kd [13–15]. We also observe two bands in that area but most of the hybridisation is seen with the 3.4 kb band. The most likely explanation for this band can be based upon the study of Gudas, *et al.* [22]. Using mouse and hamster cells, these authors have demonstrated that maturation of the TK mRNA is an ordered process and occurs in several steps. In their study, at least five different splicing intermediates (ranging from 10.2 to 1.9 kb in size) have been identified and

shown to represent different stages of intron removal. The total amount of all the immature splicing intermediates, which are formed after the poly-adenylation of the original transcript, represents about 50% of the mature mRNA which is 1.7 kb long. Lipson and Baserga [23] have also shown the accumulation of an unspliced TK hnRNA in cycling human cells and have found that it is absent in quiescent cells. Based upon these examples we believe that the 3.4 kb transcript and some of the lower bands in the H cells represent the stages in the maturation of the unspliced mRNA. The reason why so much of the message remains unspliced in the tumour cells could be related to some specific tertiary and secondary structure of the nascent TK transcripts, which favours certain specific splicosome complexes over others [22].

An alternate explanation for our observation could be that the abnormally large transcript belongs to a pseudogene of TK [24] which has become transcriptionally active, possibly as a result of insertion of a promoter [25].

In conclusion, we have demonstrated that ZAH cells express the TK gene differentially in the H and L cells and its expression correlates with the proliferative and tumorigenic potential of cells. The low level of the TK mRNA in L cells is probably due to their relatively differentiated and postmitotic status. The tumour cells also accumulate large amounts of a TK-specific RNA which most likely is the immature unspliced native transcript of the TK gene. With all these attributes the L and H subpopulations of ZAH cells offer a good model system for the study of TK gene expression.

1. Pardee AB. G1 events and regulation of cell proliferation. *Science* 1989, **246**, 603–608.
2. Fridovich-Keil JL, Hansen LJ, Keyomarsi K, Pardee AB. Progression through cell cycle: an overview. *Am Rev Resp Disease* 1990, **142**, S3–S6.
3. Baserga R, Calabretta B, Travali S, Jasulski D, Lipson KE, deRiel JK. Regulation of the expression of cell cycle genes. *Ann NY Acad Sci USA*, 1988, **551**, 283–289.
4. Pardee AB. Molecules involved in the proliferation of normal and cancer cells: presidential address. *Cancer Res* 1987, **47**, 1488–1491.
5. Coppock DC, Pardee AB. Control of thymidine kinase mRNA during cell cycle. *Mol Cell Biol* 1987, **7**, 2925–2932.
6. Hendrickson S, Wu J, Johnson LF. Cell regulation of di-hydrofolate-reductase metabolism in mouse fibroblasts. *Proc Natl Acad Sci USA* 1980, **77**, 5140–5144.
7. Jenh CH, Geyer PK, Johnson LF. Control of thymidylate synthetase mRNA content and gene transcription in an overproducing mouse cell line. *Mol Cell Biol* 1985, **5**, 2527–2632.
8. Pande G, Joshi DS, Sundaram K, Das MR. Isolation and characterisation of the two subpopulations of cells, with different lethalities from Zajdela asitic hepatoma. *Cancer Res* 1986, **46**, 1673–1678.
9. Chomoczynski P, Sacchi N. Single step method of RNA isolation by acid quanidium thiocynate phenol chloroform extraction. *Analyt Biochem* 1987, **162**, 156–159.
10. Sambrook J, Fritsch EF, Maniatis T. *A Laboratory Manual of Molecular Cloning*. New York, Cold Spring Harbor, 1989.
11. McKnight SJ, Gavis ER. Expression of the Herpes thymidine kinase gene in *Xenopus leavis* oocytes as an assay for the study of deletion mutants constructed *in vitro*. *Nucl Acid Res* 1980, **8**, 5931–5939.
12. Lee LS, Cheng YC. Human deoxy-thymidine kinase I: purification and general properties of the cytoplasmic and mitochondrial isoenzymes derived from the blast cells of acute myelocytic leukemia. *J Biol Chem* 1976, **251**, 2600–2604.
13. Flemington E, Bradshaw HDJ, Tarina-Dorge V, Denininger PL. Sequence, structure and promoter characterization of the human thymidine kinase gene. *Gene* 1987, **52**, 267–277.
14. Groudine M, Casmir C. Post transcriptional regulation of the chicken thymidine kinase gene. *Nucl Acid Res* 1984, **12**, 1427–1446.
15. Hofbauer RE, Mullner E, Seiser C, Wintersberger E. Cell cycle regulated synthesis of stable mouse thymidine kinase mRNA is mediated by a sequence within the DNA. *Nucl Acid Res* 1987, **15**, 741–752.
16. Levine A, Cantoni GL, Razin A. Inhibition of promoter activity by methylatiolation: possible involvement of protein mediators. *Proc Natl Acad Sci USA* 1991, **88**, 6515–6518.
17. Fridovich-Keil JL, Gudas JM, Dou QP, Bouvard I, Pardee AB. Growth responsive expression from the murine thymidine kinase promoter: analysis of DNA sequences. *Cell Growth Differ* 1991, **2**, 67–76.
18. Gross MK, Merrill GF. Thymidine kinase synthesis is repressed in nonreplicating muscle cells by a translational mechanism that does not affect polysomal distribution of thymidine kinase mRNA. *Proc Natl Acad Sci USA* 1989, **88**, 4987–4991.
19. Dou QP, Fridovich-Keil JL, Pardee AB. Inducible proteins binding to the murine thymidine kinase promoter in the late G_1/S phase. *Proc Natl Acad Sci USA* 1991, **88**, 1157–1161.
20. Bradley DW, Dou QP, Fridovic-Keil JL, Pardee AB. Transformed and non-transformed cells differ in stability and cell cycle regulation of a binding activity to the thymidine kinase promoter. *Proc Natl Acad Sci USA* 1990, **87**, 9310–9314.
21. Pierce GB, Speers WC. Tumors as caricatures of the process of tissue renewal: prospects for therapy by directing differentiation. *Cancer Res* 1988, **48**, 1996–2004.
22. Gudas JM, Knight GB, Pardee AB. Ordered splicing of thymidine kinase pre-mRNA during the S phase of the cell cycle. *Mol Cell Biol* 1990, **10**, 5591–5595.
23. Lipson KE, Baserga R. Transcriptional activity of the human thymidine kinase gene determined by a method using the polymerase chain reaction and an intron specific probe. *Proc Natl Acad Sci USA* 1989, **86**, 9774–9777.
24. Seiser C, Wintersberger E. Mouse thymidine kinase: sequence of a processed pseudogene. *Nucl Acid Res* 1989, **17**, 2128.
25. Hiller S, Hengstler M, Kunze M, Knippers R. Insertional activation of a promoterless thymidine kinase gene. *Mol Cell Biol* 1988, **8**, 3298–3302.