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Analysis of the rearrangements associated with carcinogen-induced activation of the hamster thymidine kinase gene

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ABSTRACT

We have previously shown that chemical carcinogen treatment of RJK92 hamster cells activates the quiescent thymidine kinase (TK) gene and that 20% of the TK+ variants have a rearrangement in the region 5′ to the TK gene (Barr et al. (1986) Mol. Cell. Biol. 6, 3023–3033). After cloning the wild type 5′ region to obtain detailed mapping data and hybridization probes, we localized the rearrangement breakpoints by Southern blot analysis to a 1.5 kb region 6 kb 5′ to the origin of transcription. This analysis also demonstrated that the rearrangements consist at least partly of a deletion of wild type sequences 5′ to this breakpoint region. The region near the transcription origin in the rearranged TK genes has a DNase I-sensitive chromatin conformation and a DNase I hypersensitive site as well as the previously described domain of demethylation (*Ibid.*). Though this domain of demethylation extends into the breakpoint region, the rearranged region is not associated with DNase I sensitivity nor hypersensitive sites. The rearrangement also does not detectably alter the growth-related regulation of TK activity in these cells.

INTRODUCTION

The recognition of chromosomal alterations in many tumors (1) has directed investigation towards potentially important steps in carcinogenesis. Structural alterations including translocations, insertions, and deletions are associated with rearrangement and altered expression of cellular genes. In lymphoid tumors, several translocations result from mistakes in the programmed rearrangement of immunoglobulin and T cell receptor genes and alter regulatory regions of c-*myc* and other putative protooncogenes (2). A second mechanism appears to be involved in the juxtaposition of c-*abl* and *bcr* genes to produce a chimeric mRNA and protein product with altered enzymatic activity in chronic myelogenous leukemia (3).

Exposure of cells to chemicals and radiation can induce chromosomal alterations as well as submicroscopic mutations (4,5). We have developed a cell culture system in which treatment of RJK92 Chinese hamster cells with the chemical carcinogen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine activates the inactive endogenous thymidine kinase (TK) gene (6). This TK gene is an example of a class of functionally intact and potentially expressible genes which are inactive because of a repressed chromosomal state (7). Southern blot analysis demonstrated that in 20% of the TK+ variants, a rearrangement occurred in the region 5′ to the TK gene (6). This inducible cell culture system presents the opportunity to explore the mechanism of carcinogen-induced gene rearrangement and the role of rearrangement in the activation of a quiescent gene. To obtain mapping data and hybridization probes in the vicinity of the rearrangements, we cloned the region 5′ to the wild type TK gene. We then used these tools to characterize the rearrangements 5′ to the TK gene. Finally, two features implicated in the control of gene expression, DNA methylation and chromatin structure, were examined to investigate the contribution of the rearranged region to the altered expression of the TK gene.

MATERIALS AND METHODS

Growth and isolation of phages

The bacterial host K802 *recA*− and the phage cloning vector lambda Charon 35 (8) were provided by F. Blattner. Bacteria were grown in NZC medium (Gibco) and preadsorbed to phage particles by mixing 0.1 ml of phage suspension, 0.1–0.3 ml of stationary phase bacterial culture, and 0.1–0.3 ml of 10 mM $MgCl_2$, 10 mM $CaCl_2$ followed by incubation at 37°C for 20 min. The multiplicity of infection for large scale liquid cultures was in the range of 10^{-3} to 10^{-2}. Phage plating, transfer to nitrocellulose, and liquid culture were otherwise performed as described (9). Phage DNA was purified by polyethylene glycol precipitation, organic extraction, glycerol step gradient

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centrifugation, proteinase K−SDS digestion, organic extraction, and dialysis (9).

Growth and isolation of plasmids

JM101 cells were transformed with plasmid pUC18 derivatives as described (10) and grown in YT medium (11). pUC18 recombinants were identified by plating in Bluo-gal (BRL) and isopropylthio-β-galactoside (BRL). For size screening, plasmid DNA was isolated by a rapid alkaline lysis procedure (12). Plasmid DNA was purified from large scale cultures by a Triton X-100 cleared lysate procedure (13) followed by ethidium bromide−CsCl equilibrium centrifugation (9).

DNA ligation and phage packaging

Prior to ligation, polylinker oligonucleotides were removed from double restriction digests of Charon 35 DNA by isopropanol precipitation (14). DNA ligations were performed overnight with T4 DNA ligase (0.1 U per μl of final reaction volume) in 50 mM Tris, pH 7.8, 5 mM $MgCl_2$, 1 mM ATP, 20 mM dithiothreitol, 50 μg/ml bovine serum albumin at 14°C. To maximize the yield of recombinant molecules, optimal concentrations of insert and vector were calculated as described (15). The product of insert and phage DNA ligation was packaged *in vitro* with the Packagene Lambda Packaging System (Promega Biotec).

DNA digestion, electrophoresis, and Southern blot analysis

Genomic DNA was isolated as described (16). DNA samples were digested with 3−10 U of restriction endonuclease per μg DNA for 2−6 h, electrophoresed in 0.5−1.5% agarose gels in Tris-acetate buffer, and blotted to nitrocellulose (9). DNA fragments were isolated from agarose by the glass powder technique (17) and nick-translated as described (18). Nitrocellulose filters were baked, prehybridized, hybridized to ^{32}P-labeled, nick-translated probes, washed, and exposed as described (18).

Chromatin analysis

RJK92 cells and derivatives were grown under standardized conditions as described (6). Twenty-four hours prior to harvest, cells were labeled with 250 nCi of [6-^3H] deoxycytidine (5−6 Ci/mmol; Moravek Biochemicals) per ml. Cells were harvested by brief trypsin treatment terminated by addition of serum-containing medium followed by two washes in cold phosphate-buffered saline. Nuclei were isolated by lysis in 0.27% Nonidet P-40 and Dounce homogenization followed by sucrose step gradient centrifugation (19). Nuclei were digested with DNase I (Boehringer-Mannheim, 4360 U/mg) and extent of digestion was determined as described (19).

Growth arrest, serum stimulation, and enzymatic assay

Cell growth was arrested by serum deprivation for 48 h in medium containing 0.1% fetal calf serum (20). Growth was then stimulated by replacement with medium containing 5% fetal calf serum. Cells were harvested by scraping and soluble extracts were prepared as described (6). TK enzymatic activity was determined by a radioisotope-DEAE filter binding assay (6).

RESULTS

Cloning and mapping the 5′ region

To facilitate cloning the 16 kb *Hind*III fragment containing the region 5′ to the wild type TK gene, 12−23 kb fragments were isolated following agarose gel electrophoresis of a *Hind*III digest of RJK92 DNA. Lambda Charon 35 was selected as the cloning vector because of its large cloning capacity, ability to propagate on *recA*$^-$ bacteria, and multiple cloning site polylinkers (8). Digestion of Charon 35 DNA with *Hind*III and *Bam*HI generated arms with *Hind*III ends and an incompatible stuffer fragment with *Bam*HI ends. After ligation of vector and insert DNA and phage packaging, we obtained a recombinant library of 6×10^5 phages.

The recombinant library was screened with probe 5-NA, a 500

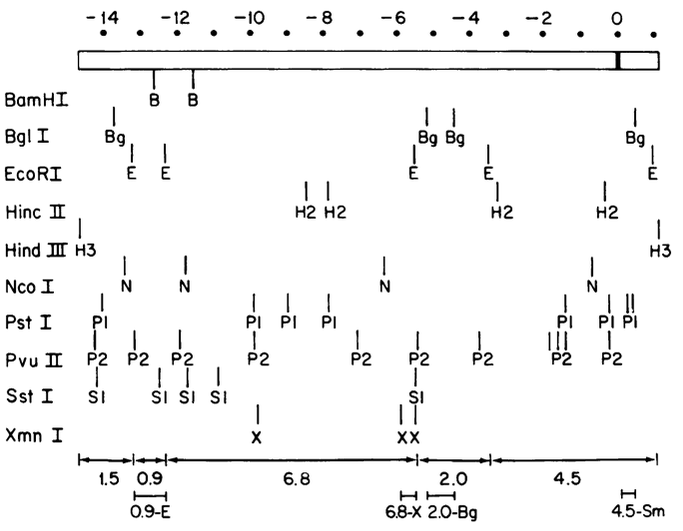

Figure 1. Map of the region 5′ to the hamster TK gene. After cloning the *Hind*III fragment into lambda Charon 35, the *Eco*RI fragments (shown as arrows) were subcloned into plasmid pUC18 and mapped. The first exon is shown as a solid box and hybridization probes are shown as line segments below the map. The numerical scale represents distances (in kb) from the origin of transcription. An abridged map of this region is shown in Fig. 3A.

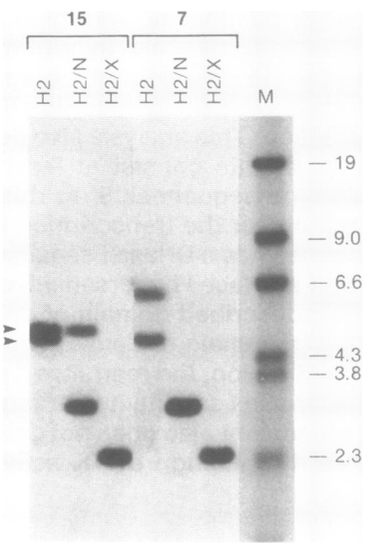

Figure 2. Southern blot analysis of the rearrangement in the region 5′ to the hamster TK gene. DNA samples (10 μg) from rearranged TK$^+$ lines 7 and 15 were digested with the designated enzymes, electrophoresed in a 0.75% agarose gel, and blotted. The blot was hybridized to probe 2.0-Bg (Fig. 1). Lane M contains an end-labeled *Hind*III-*Nco*I digest of λ DNA. The arrowheads indicate two closely spaced bands in the *Hinc*II digest of line 15 DNA. Restriction enzyme abbreviations are presented in Fig. 1.

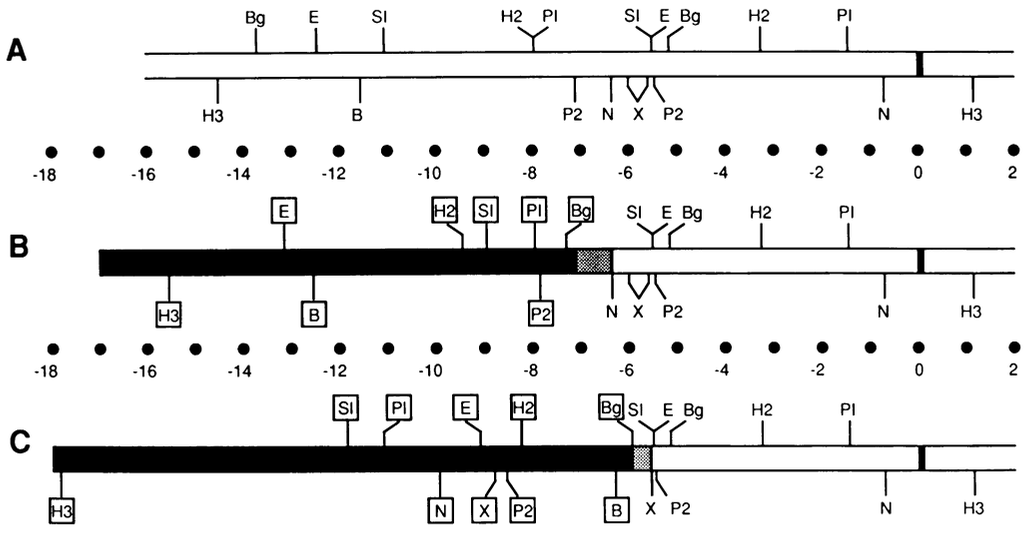

Figure 3. Comparison of the region 5′ to the TK gene in RJK92 (A) with the rearranged region in TK$^+$ lines 7 (B) and 13, 15, and 16 (C). The first exon is shown as a solid box and the numerical scale represents distances (in kb) from the origin of transcription. Restriction enzyme abbreviations are presented in Fig. 1. In the TK$^+$ variants, the rearrangement breakpoints are localized within the checkered regions and new restriction enzyme sites within the shaded rearranged regions are enclosed in boxes.

bp sequence directly 5′ to the first exon (6). After three rounds of screening, we isolated four independent recombinant phages with 16 kb inserts hybridizing to probe 5-NA. Restriction endonuclease analysis of DNA purified from large scale cultures of two of the recombinant phages revealed patterns consistent with previous mapping data (6, data not shown). These findings confirm that the recombinant phages contain the 5′ region of the hamster TK gene and that there are no detectable rearrangements in the cloned insert.

We next cloned the 6.8, 4.5, 2.0, 1.5, and 0.9 kb *Eco*RI fragments from the 16 kb insert into plasmid pUC18 (Fig. 1). Following ligation and transformation of JM101 cells, clones containing each of the five *Eco*RI inserts were identified. Sites for ten restriction endonucleases were located in each of the recombinant plasmids (Fig. 1). By comparing the maps of the plasmid inserts with initial mapping data on the 16 kb phage insert (data not shown) and previous mapping data (6), each *Eco*RI insert was located and oriented within the 16 kb insert and a complete restriction map of the region 5′ to the wild type TK gene was constructed (Fig. 1).

Localization of rearrangement breakpoints

We have previously determined that in TK$^+$ lines 7, 13, 15, and 16 the rearrangement breakpoints occurred 5′ to the *Xmn*I site at −5.8 (6), which in the cloned 5′ region has been remapped to −5.6 (the more-3′ of the two closely spaced *Xmn*I sites, Fig. 1 and 3A). To localize further these breakpoints, we examined the region between this *Xmn*I site and the *Hinc*II site at −7.9 by Southern blot analysis with probe 2.0-Bg (Fig.1). Digestion of DNA from lines 7 and 15 with *Hinc*II and *Xmn*I yields a single band pattern corresponding to a non-polymorphic diploid locus (Fig. 2). However, digestion of DNA from line 15 with *Hinc*II and *Nco*I or *Hinc*II alone generates a two band pattern corresponding to a wild type fragment and a rearranged fragment (Fig. 2). These data localize the rearrangement breakpoint between positions −5.6 and −6.35. Similarly, the conversion from a one band pattern with *Hinc*II and *Nco*I digestion of line 7 DNA to a two band pattern with *Hinc*II alone (Fig. 2) indicates

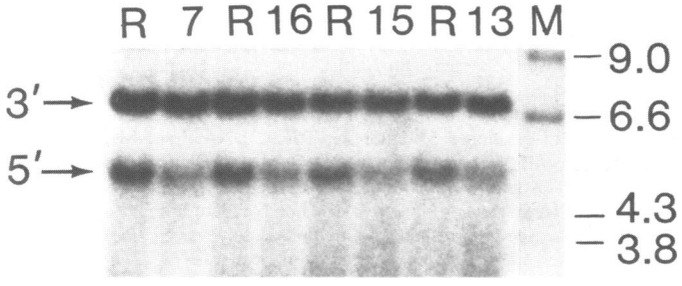

Figure 4. Comparison of the copy number of wild type sequences 5′ and 3′ to the rearrangement breakpoint region. DNA samples (10 μg) from RJK92 (R) and rearranged TK$^+$ lines 7, 13, 15, and 16 were digested with *Hind*III and *Xmn*I, electrophoresed in a 0.75% agarose gel, and blotted. The blot was hybridized to probe 2.0-Bg (to detect wild type sequences 3′ to the breakpoint region) and probe 0.9-E (to detect wild type sequences 5′ to the breakpoint region). Lane M contains an end-labeled *Hind*III-*Nco*I digest of λ DNA. The locations of the probes are shown in Fig. 1.

that the breakpoint in line 7 is between −6.3 and −7.9. Digestion of DNA from RJK92 with these restriction endonucleases generates only single band patterns (data not shown).

After further Southern blot analysis of these cell lines with probe 6.8-X (Fig. 1), we constructed maps of the rearranged 5′ regions in lines 7, 13, 15, and 16 (Fig. 3). In line 7, the loss of the *Pvu*II site at −7.1 localizes the breakpoint to the 750 bp fragment between −6.35 and −7.1. In lines 13, 15, and 16, the occurrence of a new *Bgl*I site at −5.95 localizes the breakpoint to the 350 bp fragment between −5.6 and −5.95. Probe 6.8-X contains the region in which this latter breakpoint occurs. Because hybridization with probe 6.8-X under stringent conditions demonstrates a discrete banding pattern (Fig. 5 and 6 and data not shown), we conclude that this rearrangement occurs within a single copy sequence.

This Southern blot analysis does not demonstrate any differences among the rearrangements in lines 13, 15, and 16 (data not shown). These three lines were derived from a common carcinogen-treated pool of RJK92 cells (6) and may represent

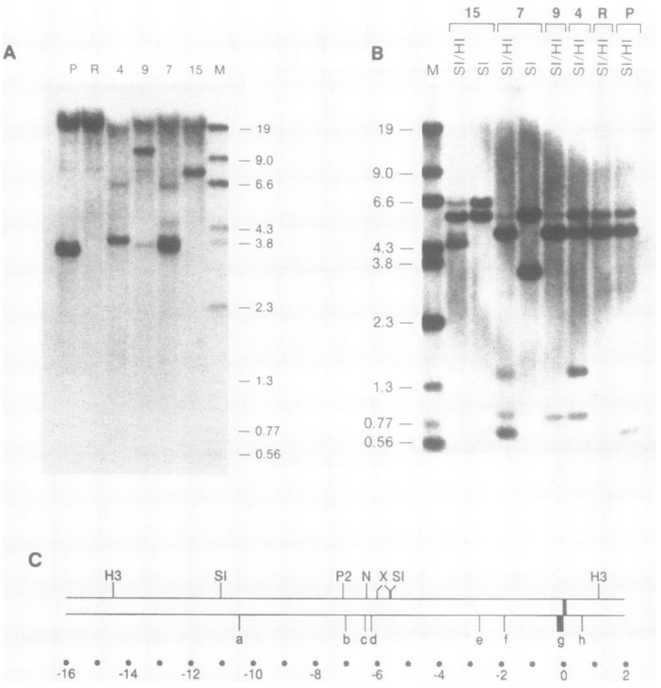

Figure 5. Methylation analysis of the region 5′ to the TK gene. DNA samples (10 μg) from RJK92 (R), unrearranged TK$^+$ lines 4 and 9, and rearranged TK$^+$ lines 7 and 15 were digested with *Hha*I (A) or *Sst*I (S1) and *Hha*I (H1) (B), electrophoresed in a 1.25% (A) or 0.9% (B) agarose gel, and blotted. Both blots were hybridized to probe 6.8-X (Fig. 1). Lane P is a digest of a mixture of phage DNA containing the cloned 5′ region and RJK92 DNA. Lane M contains an end-labeled *Hind*III-*Nco*I digest of λ DNA. (C) Map of *Hha*I sites in the region 5′ to wild type TK gene. *Hha*I sites a−e were located by Southern blot analysis of genomic DNA and sites f−h were located on ethidium bromide-stained gels of cloned DNA. Site g corresponds to multiple closely spaced *Hha*I sites. The first exon is shown as a solid box and the numerical scale represents distances (in kb) from the origin of transcription. Restriction enzyme abbreviations are presented in Fig. 1.

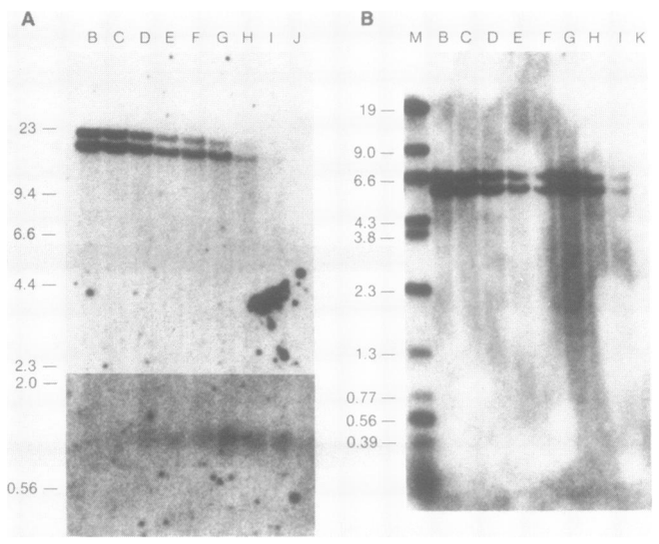

Figure 6. Chromatin analysis of the region 5′ to the TK gene in rearranged TK$^+$ line 15. Nuclei were prepared and digested with 0−60 U of DNase I for 4 min. (A) To examine the region near the origin of transcription, DNA samples (10 μg) were digested with *Hind*III, electrophoresed in a 0.6% agarose gel, and blotted; the blot was hybridized to probe 4.5-Sm. The lower panel was exposed twice as long as the top panel. (B) To examine the rearranged region, DNA samples (10 μg) were digested with *Sst*I, electrophoresed in a 0.9% agarose gel, and blotted; the blot was hybridized to probe 6.8-X. Lane M contains an end-labeled *Hind*III-*Nco*I digest of λ DNA. The locations of the probes are shown in Fig. 1. (Extents of digestion: B—0.2%, C—0.3%, D—0.6%, E—0.7%, F—1.4%, G—1.3%, H—1.4%, I—2.6%, J—4.6%, K—9.5%).

subclones of a common rearranged TK$^+$ variant. However, these findings may also indicate the presence of a rearrangement hot spot.

Deletion analysis

To investigate the involvement of a deletion in the rearrangement event, we isolated probe 0.9-E from the wild type region 5′ to the rearrangement breakpoints (Fig. 1); this probe hybridizes to a 4.8 kb *Hind*III-*Xmn*I fragment. The hybridization intensity of this fragment located 5′ to the breakpoints was compared to the intensity of the 6.6 kb *Hind*III-*Xmn*I fragment located 3′ to the breakpoints and detected by probe 2.0-Bg (Fig. 1 and 4). The intensity of the 3′ fragment was the same in both parental and rearranged cell lines (Fig. 4). In contrast, the intensity of the 5′ fragment was reduced by approximately 50% in each rearranged cell line relative to the parental line (Fig. 4). Therefore the rearrangements consist at least partly of a deletion of wild type sequences 5′ to the rearrangement breakpoint region.

Methylation analysis

We have previously demonstrated that carcinogen-induced TK expression is correlated with demethylation in the region near the origin of transcription and, in rearranged cell lines, the rearranged TK gene copy is demethylated (6). To investigate the relationship between rearrangement and demethylation, we determined whether the domain of demethylation extends into the breakpoint region. DNA samples from parental TK$^-$ line RJK92, rearranged TK$^+$ lines 7 and 15, and unrearranged TK$^+$ lines 4 and 9 were digested with the methylation-sensitive restriction endonuclease *Hha*I and the Southern blot was hybridized with probe 6.8-X (Fig. 5A). For an unmethylated control, we digested a mixture of phage DNA containing the cloned 16 kb 5′ insert and RJK92 DNA (Fig. 5A and B, Lane P). In all TK$^+$ lines, there are low molecular weight bands corresponding to demethylated copies of the 5′ region as well as a high molecular weight band corresponding to highly methylated copies (Fig. 5A). Therefore, the domain of demethylation extends into the breakpoint region, but this extension does not distinguish between rearranged and unrearranged TK genes.

These sites of demethylation were mapped by digestion with *Hha*I and methylation-insensitive restriction endonuclease *Sst*I (Fig. 5B). Following hybridization with probe 6.8-X, three *Hha*I sites labeled b, c, and d (Fig. 5C) were detected in the rearrangement breakpoint region. Demethylation of some or all of these sites, corresponding to 1.45, 0.85, and 0.65 kb bands, correlates with TK gene expression (Fig. 5B). In line 4, sites b and c are demethylated while in line 9, site c is demethylated and in line 7, sites b, c, and d are demethylated. Because site b is detected in unrearranged line 4 and rearranged line 7, the rearrangement breakpoint in line 7 probably occurs directly 5′ to site b. However, we cannot exclude that some of these demethylated sites in line 7 were introduced by the rearrangement and fortuitously produced similarly sized bands. The rearrangement in line 15 removes the region containing sites b, c, and d and a new demethylated *Hha*I site is detected within

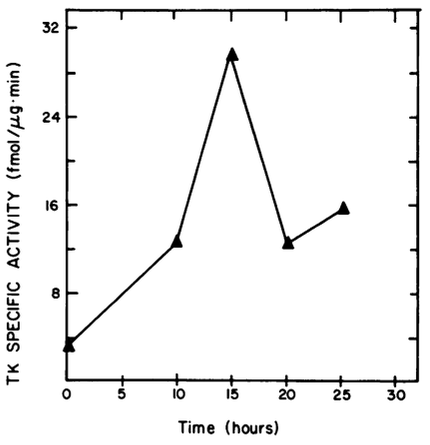

Figure 7. Growth-related regulation of TK enzyme activity in rearranged TK+ line 15. Following growth arrest for 2 days in medium containing 0.1% fetal calf serum, the medium was replaced with 5% serum-containing medium at time = 0 h. Cells were harvested and soluble extracts were prepared at the indicated time points. Each extract was assayed for TK enzymatic activity.

the rearranged region 4.4 kb 5′ to the *Sst*I site (Fig. 5B).

Upstream of sites b, c, and d, we have detected *Hha*I site a (Fig. 5C) whose demethylation does not correlate with TK expression (Fig. 5B). Demethylation at this site and infrequent demethylation of downstream sites account for the faint low molecular weight bands in the *Hha*I digest of RJK92 DNA (Fig. 5A, Lane R). In RJK92 cells, site a is demethylated in a subset of gene copies (Fig 5B, Lane R); the 5.7 and 4.8 kb bands correspond to copies with methylated and unmethylated site a, respectively. The persistence of both the 4.8 and 5.7 kb bands in digests of the TK+ lines indicates that demethylation of sites b, c, and d and gene activation cannot be simply localized to one of these two classes of gene copies.

Digestion of DNA samples from lines 7 and 15 with *Sst*I permits the rearranged and wild type gene copies to be distinguished (Fig. 5B). Digestion of the two band *Sst*I pattern in line 7 with *Hha*I results in disappearance of the lower *Sst*I band corresponding to the rearranged copy. The higher *Sst*I band corresponding to the wild type copy shifts to the position of wild type copies with demethylated site a. Therefore the rearranged copy in line 7 is the copy demethylated at sites b, c, and d. In line 15, the higher rearranged *Sst*I band is sensitive to *Hha*I digestion while the lower wild type *Sst*I band is resistant. This result again indicates that the rearranged copy is involved in the demethylation and activation processes.

*Hha*I and *Sst*I digestion of line 15 DNA did not result in complete disappearance of the higher *Sst*I band (Fig. 5B). This result was reproducible with different concentrations of *Hha*I (data not shown) and thus cannot be explained by incomplete digestion. We conclude that some copies of the rearranged gene are not demethylated at this *Hha*I site in the rearranged region. When DNA samples from higher passages of the same cell lines were digested, identical results were obtained (data not shown) indicating that there was no detectable drift in the methylation patterns with increasing passage. This heterogeneity may arise early in the life of these cell lines and then be stably inherited. Heterogeneity in the methylation status of certain sites was also noted in our methylation analysis of the region near the transcription origin (6).

Chromatin analysis

Investigations of the chromatin structure of many genes have demonstrated an association of DNase I sensitivity and hypersensitive sites with gene activity (19,21,22). We used the enzyme DNase I to probe the chromatin structure and thereby search for potentially functional elements in the region 5′ to the TK gene in TK− RJK92 cells, unrearranged TK+ line 9, and rearranged TK+ lines 7 and 15. Following digestion of nuclei with varying concentrations of DNase I, we isolated DNA, digested with *Hind*III, and hybridized the Southern blots with probe 4.5-Sm (Fig. 1). This combination of probe and restriction endonuclease permits analysis of a large region extending from the first intron into the breakpoint region as well as the rearranged region in lines 7 and 15. In TK+ lines 7, 9, and 15, we detected a 1.15 kb fragment which corresponds to a DNase I hypersensitive site mapping near the origin of transcription (Fig. 6A, data not shown for lines 7 and 9). This hypersensitive site was not detected in TK− line RJK92 (data not shown). The occurrence of this hypersensitive site therefore correlates with TK gene expression. In contrast to the region near the transcription origin, we did not identify hypersensitive sites which map within the rearranged region in lines 7 and 15.

The overall DNase I sensitivity of the wild type and rearranged gene copies could also be compared in these experiments. With increasing extents of DNase I digestion, the intensity of the 19 kb fragment corresponding to the rearranged gene copy in line 15 decreased more rapidly than that of the 16 kb fragment corresponding to the wild type copy (Fig. 6A). The rearranged gene copy in line 7 was similarly sensitive to DNase I digestion (data not shown). Therefore the presence of rearrangement and TK gene expression correlates with increased DNase I sensitivity. As suggested in our analysis of DNA methylation, these findings support the conclusion that the gene rearrangements 5′ to the TK gene are associated with TK gene activation.

We next investigated whether DNase I sensitivity occurred within the rearranged region by digesting DNA samples from DNase I-treated nuclei with *Eco*RI (line 7) or *Sst*I (line 15) and hybridizing the Southern blots with probe 6.8-X (Fig. 6B, data not shown for line 7). As found in the previous experiment (Fig. 6A), no hypersensitive sites were detected within the rearranged region (Fig 6B). Furthermore, in this region, the higher molecular weight fragment corresponding to the rearranged copy is equally sensitive to DNase I as the lower molecular weight fragment corresponding to wild type copy. The domain of DNase I sensitivity therefore does not extend into the rearranged region. We conclude that the DNase I sensitivity detected in the previous experiment resulted from altered chromatin structure in the vicinity of the origin of transcription.

Regulation of TK activity

Investigations of normal TK expression have demonstrated regulation by growth-related signals (20.23.24). Low or absent TK activity is associated with the quiescent serum-arrested state; following serum stimulation, TK activity increases as cells enter S phase. In normal cells, c-*myc* expression is also regulated by growth-related signals but, following a variety of rearrangements, regulation of c-*myc* becomes constitutive (2). To determine whether TK expression still responds to normal regulatory signals following carcinogen-induced rearrangement, we studied the regulation of TK enzyme activity in line 15. After incubation of cells for 2 days in medium with 0.1% serum, the TK activity was less than 4 U (Fig. 7). Following stimulation with 5% serum,

the enzyme activity increased and peaked at 15 hours. This profile is similar to that of cell lines without a rearrangement 5′ to the TK gene (data not shown). Therefore, the rearrangement does not abolish the normal growth-related regulation of TK activity. This finding is consistent with evidence that much of this regulation occurs at the posttranscriptional level (23,24).

DISCUSSION

To explore the structural basis and molecular consequences of carcinogen-induced gene rearrangement, we have analyzed the 5′ rearrangements associated with carcinogen-induced activation of the quiescent hamster TK gene. Southern blot analysis demonstrated that the rearrangement breakpoints occur in a region approximately 6 kb 5′ to the origin of transcription. Furthermore, the rearrangement includes a deletion of wild type sequences 5′ to this breakpoint region. TK gene activation is associated with demethylation, a DNase I-sensitive chromatin conformation, and a DNase I hypersensitive site in the region adjacent to the transcription origin. The domain of demethylation extends into the breakpoint region but the rearranged region is not associated with DNase I-sensitive chromatin nor hypersensitive sites. In addition, this rearrangement does not detectably alter the growth-related regulation of TK activity.

We have identified two distinct carcinogen-induced rearrangements in the region 5′ to the TK gene. The common rearrangement breakpoint in cell lines 13, 15, and 16 and the distinct breakpoint in line 7 both occur within a 1.5 kb region. Clustering of rearrangement breakpoints has also been observed in studies of human tumors such as chronic myelogenous leukemia in which there is a 5.8 kb breakpoint cluster region within the *bcr* gene (3). These clustering regions may identify DNA or chromatin structures predisposing the gene to rearrangement. Alternatively, these breakpoint clusters may signify the presence of regulatory or coding regions which must be disrupted to effect the alteration in gene expression or flanking elements which must be spared to maintain essential activity.

Comparison of the copy number of wild type sequences 5′ and 3′ to the breakpoint region revealed a deletion of 5′ wild type sequences in the rearranged TK^+ cell lines. We have only formally demonstrated deletion of sequences corresponding to probe 0.9-E. However, because of the occurrence of this deletion in all rearrangements and the 6 kb distance between probe 0.9-E and the breakpoints, we suggest that a larger deletion is the most likely basis for the rearrangement. Measuring from the breakpoint to the most-5′ restriction site detected, the deletion would be at least 12 kb in lines 13, 15, and 16 and 8.5 kb in line 7. This deletion could be the result of either intrachromosomal or interchromosomal recombination so that the rearranged region could originate from anywhere in the genome.

The association between the 5′ rearrangement and TK gene activation is supported by the localization of demethylation, DNase I-sensitive chromatin, and a DNase I hypersensitive site to the rearranged copy of the TK gene in lines 7 and 15. Still, we cannot distinguish between cause and effect. Carcinogen treatment could cause rearrangement which in turn induced gene activation or alternatively carcinogen treatment could directly activate gene expression which then facilitated gene rearrangement.

If rearrangement caused gene activation, one possible mechanism is the juxtaposition of strong positive regulatory sequences or other features of an active chromatin domain adjacent to the inactive TK gene. These elements have been implicated in the deregulation of c-*myc* expression by translocation in Burkitt's lymphoma (2). Previous studies have established that many if not all cis-activating transcriptional elements, such as enhancers and promoters, are associated with DNase I hypersensitive sites (21). In our study, the ability to detect hypersensitive sites is demonstrated by the finding of a site in the vicinity of the known promoter of the hamster TK gene (25); a similar site has been reported in the chicken TK promoter (23). Our analysis failed to reveal any hypersensitive sites within the rearranged region. Furthermore, the rearranged region is not organized into a DNase I-sensitive conformation and hence does not demonstrate the features of an active chromatin domain. Therefore, our findings do not support the presence of activating elements within the examined portion of the rearranged sequence.

Another possible mechanism for activation is the removal of negative cis-regulatory elements. Sequences termed silencers have been identified in flanking regions of yeast and mammalian genes (26,27). Recent evidence has demonstrated that DNase I hypersensitive sites are often associated with silencers in cells which do not express the associated gene (21). Our chromatin analysis of the 5′ flanking region of the TK gene in RJK92 cells did not reveal any such hypersensitive site and thus does not support this mechanism of activation.

Our findings indicate an interesting relationship between methylation and chromatin in these TK^+ cells. Investigations of other genes have correlated demethylation in the 5′ region and conversion to DNase I-sensitivity with gene activity (22,28). A study of the chicken α-globin locus demonstrated coincidence of the domains of demethylation and DNase I sensitivity in erythrocytes expressing this gene (29). The ability of DNA methylation to influence chromatin assembly was suggested by experiments in which *in vitro* methylated and unmethylated sequences transfected into cultured cells formed DNase I-resistant and sensitive structures, respectively (30). In our rearranged TK^+ variants, we also observed correlation of demethylation and DNase I sensitivity near the transcription origin. The extension of the demethylation domain into the breakpoint region without extension of the DNase I-sensitive domain indicates that demethylation is not sufficient to dictate assembly of an active chromatin domain. These findings are consistent with hypotheses of carcinogen damage leading to demethylation, either directly or following rearrangement. Such demethylation would be nonspecific and affect sequences other than those directly correlated with gene activity. Therefore, only a subset of this domain of demethylation might be needed, in conjunction with other factors, to direct assembly of an active chromatin domain.

Our analysis of rearrangement associated with carcinogen-induced TK gene activation presents a case of rearrangement differing from the well characterized examples in hematopoietic tumors (2,3). As a result of our findings, we suggest the possibility of alternative roles for rearrangement in altered gene expression. Rearrangement alone may be a sufficient perturbation to destabilize a repressed gene locus or perhaps rearrangement can be the result rather than the cause of gene activation. Investigation of other experimental and tumor-related gene rearrangements will expand the repertoire of possible relationships between rearrangement and altered gene expression.

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