Hepatoma variants (C2) are defective for transcriptional and post-transcriptional actions from both endogenous and viral genomes

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A series of rat hepatoma cell lines was infected with a recombinant adenovirus bearing the rat albumin promoter. Transcription from this promoter was scored directly and was highest in FAO, the differentiated parent, undetectable in C2, a cell variant that has lost almost all hepatocytic characteristics, and high again in C2-Rev7, a 'revertant' cell line derived from C2 that has regained the ability to produce many proteins characteristic of hepatocytes. The endogenous albumin gene is not transcribed in C2 cells, and at a very low rate in C2-Rev7 cells, which accumulate endogenous albumin mRNA at close to normal amounts. Thus the C2-Rev7 'recovery' of albumin mRNA concentration for the endogenous gene is based mainly on post-transcriptional events while the ability of C2-Rev7 to transcribe the albumin promoter in the viral genome is based on a transcriptional factor(s). We also showed that the C2 phenotype included post-transcriptional effects for other genes: transcription of phenylalanine hydroxylase and phosphoenolpyruvate carboxykinase mRNA sequences continue in C2 at rates equivalent to FAO but these C2 cells have no mRNA for these proteins while FAO does. In addition, C2 cells transcribed certain early adenovirus transcription units (E2 and 4) as well as FAO cells but accumulated E2 mRNAs poorly if at all. The changes that led to the C2-Rev7 cell line produced a return to normal of the ability to accumulate these viral mRNAs. Thus a major event in the C2 to C2-Rev7 transition involves post-transcriptional processes as well as the ability to transcribe the albumin promoter positioned in the virus genome.

Key words: albumin gene transcription/post-transcriptional gene control/variant hepatoma cells

Introduction

The basis of tissue-specific gene expression is being explored widely by the introduction into cultured, partially differentiated cells of promoters and enhancers elements taken from genes that are normally expressed in one or a few cell types. Well before the developments that made possible the construction of recombinant DNA molecules and the introduction of reconstructed genes into cells, Weiss and her colleagues had begun to examine variant cell lines in an effort to examine cellular changes that affected expression of those genes known to be active in hepatocytes (Deschatrette and Weiss, 1974; Deschatrette *et al.*, 1980). In this paper we combine these two approaches: non-replicating, recombinant adenoviruses containing sequences from the rat albumin gene (Babiss *et al.*, 1986; Friedman *et al.*, 1986) were used to infect several rat cell lines originally derived from the Reuber rat hepatoma (Deschatrette and Weiss, 1974; Deschatrette

et al., 1980). These cell lines are known to express a group of liver-specific proteins, including albumin, at greatly differing rates. For example the cell line FAO produces albumin at one-third to one-half the rate of hepatocytes; C2, a derivative of FAO does not produce albumin and C2-Rev7 makes albumin at about one-third the rate of FAO (Clayton *et al.*, 1985). We previously examined the basis for these variations and found that FAO, although containing ~50% as much albumin mRNA as hepatocytes transcribed only ~10% as much RNA. C2 lacked albumin mRNA and had no detectable transcriptional signal. C2-Rev7, while recovering substantial albumin mRNA, had a barely detectable transcriptional signal (Clayton *et al.*, 1985). Thus the levels of albumin expression observed in these hepatoma cell lines were not explained completely by co-ordinate changes in transcription. In addition to albumin, several other genes were transcribed in C2 without being represented in mRNA. Because regulation of gene expression in these cells obviously lies both at the transcriptional and at the post-transcriptional level, examining the function of recombinant genes in these cells demands that direct analysis of transcription rates and not analysis of mRNA levels be carried out to determine whether the control of a particular gene lies at the transcriptional or post-transcriptional level. Using recombinant adenovirus containing the rat albumin promoter (Babiss *et al.*, 1986; Friedman *et al.*, 1986) we have been able to assay transcription from this promoter directly and have also been able to score steady-state levels of recombinant mRNAs. A previous report (Ott *et al.*, 1984) described enzyme production from a transfected recombinant plasmid containing the albumin promoter driving the bacterial chloramphenicol acetylase (CAT) gene in these cell lines (Ott *et al.*, 1984). While transfection of FAO, C2 and C2-Rev7 cells was poor compared to other cell lines it was found that CAT formation was detectable in FAO and C2-Rev7 but not C2 cells. However the level of gene regulation in these experiments could not be determined.

We have indeed found that the albumin promoter within the adenovirus genome drives transcription best in FAO cells, not at all in C2 cells and in C2-Rev7 cells at a rate about one-half that of FAO. Thus there is an understandable variation in recombinant albumin mRNA concentrations based in part on the presence of one or more transcription factors in FAO and C2-Rev7 that are lacking in C2. These factors, which are sufficient for activity of the viral albumin promoter in C2-Rev7 cells, are not, however, sufficient for an equivalent transcriptional activation of the endogenous albumin gene which is only ~2% as active in C2-Rev7 as in FAO.

Furthermore, there is evidence in the adenovirus-infected cells that the event(s) leading to the C2 variation affect not only liver-specific gene transcription in a complex manner but also affect mRNA accumulation of some cellular and viral mRNAs. The early viral E2 and E4 genes are transcribed similarly in all three cell types but diminished levels of these viral mRNAs accumulate in C2, particularly for the viral E2 mRNA. Thus the C2 state affects the transcription of some but not all liver-specific genes

and also affects accumulation of several different mRNAs as well. The C2-Rev7 cell has partially recovered the transcriptional abilities of FAO and apparently has fully recovered the ability to accumulate mRNAs. In fact C2-Rev7 seems to be a 'super-accumulator' for albumin mRNA from the endogenous gene since it transcribes poorly but accumulates equally compared to FAO cells.

Results

To test whether the hepatoma cell lines that accumulate rat albumin mRNA (C2-Rev7 and FAO cells) start transcription of the endogenous albumin gene at the same site as hepatocytes, a 5' assay for rat albumin mRNA was developed (Friedman *et al.*, 1986). In this assay a labeled SP6 RNA antisense probe is protected from nuclease digestion for a stretch of 75 nucleotides by the first exon of the rat albumin mRNA. FAO and C2-Rev7 cells, but not C2 cells, contained albumin mRNA in similar amounts and this mRNA started at the same site (Figure 1) as had previously been shown for hepatocytes (Babiss *et al.*, 1986; Friedman *et al.*, 1986). Thus presumably at least some of the same transcription factors might be available in hepatocytes, FAO and C2-Rev7 cells.

Fig. 1. Determination of the albumin cap site in FAO and C2-Rev7. 1 μ g of total RNA was hybridized to an anti-sense DNA generated from a *Hpa*II (+74)–*Hind*III (–441) subclone of the rat albumin gene (Sargent *et al.*, 1981; Friedman *et al.*, 1986). T2 RNase-resistant hybrids were electrophoresed on a 5% urea acrylamide gel. The gel was dried and a film was exposed for 12 h.

Fig. 2. Genomic map of alb454. The preparation of this virus has been described by Babiss *et al.* (1986). The adenovirus genome is drawn with emphasis on the left end of the virus where a deletion between nucleotides 454 and 3320 was replaced with a subgenomic fragment of the rat albumin gene extending from –441 to +947 bp (dotted line). The virus retains the E1A enhancer sequences between nucleotides 194 and 454 but lacks entire E1A regions including the promoter and lacks the E1B promoter and first exon. The two boxed regions represent the first two exons of the albumin gene, and the fusion RNA between these two exons and the E1B second exon is represented by the line above the genomic map.

Fig. 3. Transcription assays in FAO, C2 and C2-Rev7 cells. FAO, C2 and C2-Rev7 cells were infected for 24 h with the alb454 virus, and labeled nuclear RNA was extracted after nascent chain elongation in the presence of [α - 32 P]UTP. Labeled RNA was hybridized to dots of DNA (5 μ g/dot) on nitrocellulose filters, unpaired RNA was digested with RNase and hybrids were detected by autoradiography. The viral albumin transcription unit is detailed on the bottom panel which illustrates that the viral albumin transcripts continue from rat albumin sequences into E1B sequences which are scored by three separate cloned E1B DNA fragments. Other cloned DNAs used include actin, r.alb (a 1-kb segment of the rat albumin gene downstream of the albumin fragment included in the recombinant virus), phenylalanine hydroxylase (P-OH), phosphoenolpyruvate carboxykinase (pepCk), as well as the viral E2 and E4 probes. pBR and pUC are plasmid DNAs without inserts.

To assess the transcription signals of endogenous genes expressed in hepatocytes compared to recombinant genes containing the albumin promoter, each of these cell lines was infected with a recombinant adenovirus termed alb454 (Figure 2). This virus lacks the E1A gene, E1B promoter and a portion of the E1B first exon included in the mature 22S mRNA (Babiss *et al.*, 1986). In their place has been inserted a 1398-bp segment of the rat albumin gene extending from -441 to +957. The deletion in the virus genome extends from adenovirus nucleotide 454 to 3320. Thus the alb454 virus includes the E1A enhancer sequences between nucleotides 194 and 454 (Babiss *et al.*, 1986; Friedman *et al.*, 1986). In the recombinant virus, the albumin promoter directs the synthesis of two species of mRNAs in hepatoma cells containing the first two exons of rat albumin fused to either the first or second exons of E1B (thus there are two E1B bands protected in the Sp6 assay shown in Figure 4). Transcription of the viral albumin gene can be scored by infecting hepatoma cells with this recombinant adenovirus and measuring E1B transcription. Transcription of the endogenous albumin gene can be measured simultaneously by scoring transcription of a segment of the albumin gene downstream of the albumin gene fragment that is present in the virus. Transcription of other endogenous genes can be scored with other available probes.

In the first experiment FAO, C2 and C2-Rev7 cells were infected with 20 p.f.u./cell of the alb454 virus for 24 h and transcription assays carried out for a number of genes. The transcription rate of the endogenous albumin gene (dot marked r.alb) was high in FAO cells and undetectable in C2 cells. Transcription of the endogenous gene was definitely detectable in C2-Rev7 cells but only at a level that was 2–5% of FAO cells. Transcription of the endogenous PepCK and phenylalanine hydroxylase (PH) genes also occurred in all three cell lines as did transcription of the actin gene. Some of these results confirm and extend those of previously published work (Clayton *et al.*, 1985). In the present experiments more radioactive RNA was used and the low but definite level of endogenous albumin gene transcription in C2-Rev7 can be seen as well as a definite signal for the PH gene in all cell lines. Again as in the past work,

the PepCK signal is seen to be similar (within a factor of two) in all cell lines. These transcription results are remarkable because C2 has no mRNA for either PepCK or PH, and C2-Rev7 has an equal amount of albumin mRNA with only 2–5% the transcription signal of FAO cells (Figures 1 and 3 and Clayton *et al.*, 1985).

Turning to the analysis of transcription directed by the albumin promoter located on the viral genome (scored by hybridization to DNA from the E1B region) we found the signal to be strongest in FAO cells. Since the E1B probe (SB6) is only about one quarter the length of the r-alb probe, the viral promoter appeared to be about as active as that of the endogenous gene in FAO cells. It is unclear, however, how many of the viral DNA molecules are transcriptionally active (20 p.f.u./cell was the added multiplicity of infection). In C2 cells the viral albumin promoter did not direct transcription at a level above background. However, in C2-Rev7 cells the viral albumin promoter was ~30% as active as in FAO cells and the signal was considerably stronger than for the endogenous gene in this cell line. Thus the viral promoter was handled qualitatively in a similar way as the endogenous promoter (FAO > C2-Rev7 > C2) but the C2-Rev7 cells transcribed the viral promoter at a quantitatively greater rate relative to the endogenous gene.

All three cell types gave similar results for the transcription of viral early genes indicating the viral DNA entered and was transcribed equally in the nuclei of all three cell types. (The different signal strengths for the two viral genes E2 and E4 in Figure 3 reflect mainly different probe sizes; the E4 probe was 2 kb and the E2 probe 0.4 kb. The data for E2 were from a separate experiment in which no background signal was detectable.)

Levels of albumin mRNA generated from the viral promoter in different cell types

Next, the levels of steady-state RNAs from the viral transcription units were scored using RNA protection assays with a series of different single-stranded labeled RNA probes specific for the viral 3' exon of the fused albumin-E1B mRNA (Friedman *et al.*, 1986), for the viral E2 and E4 regions and for the actin gene as a control.

Fig. 4. Steady-state RNAs in infected FAO, C2 and C2-Rev7 cells. Each of these cell lines were infected with 20 p.f.u./cell of the alb454 virus and assayed for the presence of (a) E1B (viral albumin), (b) E2, (c) E4, (d) actin RNAs using RNase protection assays. Either 10 μ g (E1B, E2, E4) or 1 μ g (actin) total RNA was hybridized to anti-sense labeled RNA probes for each of these transcription units. T2 RNase-resistant hybrid were electrophoresed and autoradiographic exposures were for 12–48 h. The length of the protected RNA probe was determined by comparison to labeled DNA markers and this length is shown to the right of each exposure.

As had been shown to be the case for the endogenous gene (Figure 1), equivalent levels of the albumin-E1B fusion RNAs were present in infected FAO and C2-Rev7 cells (Figure 4a). No E1B RNA could be detected in C2 cells with short exposures and only very faint signals after a 20-fold longer exposure. The total mRNA recovered from C2 cells was intact because near equivalent levels of actin RNA were observed for all three cell lines (Figure 4d). We were not able to map directly the RNA start site of the albumin RNA coming from the virus promoter because the exogenous and endogenous albumin genes share the same 5' end. We have previously shown however that the transcription machinery in human hepatoma cells and mouse hepatocytes utilize the correct albumin gene start-site in the recombinant viral DNA under many different circumstances (Babiss *et al.*, 1987). Further, it seems reasonable that rat hepatoma cells might use the same start on the viral DNA as they do on the cell gene (Babiss *et al.*, 1986; Friedman *et al.*, 1986).

mRNA production from early viral transcription units in FAO, C2 and C2-Rev7 cell lines

As shown in Figure 2 the transcription rates of the early viral genes E2 and E4 were similar in all three cell types. However, the steady-state RNA levels for each of these genes gave a very different picture. Very low levels (2–5%) of E2 mRNA were present in C2 cells relative to FAO and C2-Rev7 cells. Also, there was ~5-fold less E4 RNA relative to the other two cell types (Figure 4b,c). The levels of actin RNA in the same samples were similar (within a factor of two). Thus, there was a marked difference in the post-transcriptional fate of the early viral RNAs, in C2 cells compared to the other two cell lines.

Discussion

The FAO cell line is a 'differentiated' hepatoma line that synthesizes a number of proteins known to be specific products of hepatocytes (Deschatrette and Weiss, 1974; Deschatrette *et al.*, 1980). In addition eight out of nine liver-specific cDNA probes detected mRNAs in FAO (Clayton *et al.*, 1985). C2 cells were selected as morphological variants from the FAO line. (FAO cells usually have a cuboidal hepatocyte like-appearance.) From the C2 line, C2-Rev7 cells were developed in glucose-free medium, demanding that gluconeogenesis, a liver function in mammals, supply carbohydrate for energy derivation. How many and what type of changes occurred that produce the C2 and C2-Rev7 phenotypes remains unknown.

In this and in previous studies we have found two phenotypic characteristics of mRNA metabolism in C2 cells that are at least partially reversed in C2-Rev7 cells: C2 cells no longer transcribe the endogenous albumin gene; likewise, C2 does not actively transcribe the albumin promoter on the adenovirus genome whereas FAO and C2-Rev7 both do. C2 cells therefore have definitely lost some trans-acting transcriptional capacity which could reflect the disappearance of an activator or the appearance of a repressor in these cells. The reduction of the albumin transcription rate in C2 cells is not likely to be due to the appearance of a repressor because fusion of C2 cells to Fv-5, an albumin-producing hepatoma cell, produces heterokaryons that continue to express albumin (Cassio *et al.*, 1981). Recent studies with deletions of the albumin promoter suggest that positive acting transcription factors exist in hepatoma cells that in part control the activity of the albumin gene by binding to the DNA in the region between -90 and -120 (Babiss *et al.*, 1986; Babiss, Herbst and Darnell, in preparation; M.Yaniv, personal com-

munication). No evidence of a repressor binding site was detected in this work.

The second phenotypic change in C2 cells is that they do not accumulate certain mRNAs although nuclear synthesis of these mRNA sequences occurs. This phenotypic characteristic applies to endogenous mRNAs, PepCK and PH, both normally specific products of hepatocytes as well as to early viral E2 mRNA from the adenovirus chromosome. All of these genes are transcribed in C2 cells as well as in FAO cells but almost no detectable mRNA from any of these genes was found in the cytoplasm of C2 cells (Clayton *et al.*, 1985). Of course the cells grow and do accumulate some cell mRNAs, e.g. actin. Thus the failure to express liver-specific genes in the C2 state is not due only to transcriptional changes compared to FAO but in changes in the ability to accumulate specific mRNAs from apparently active transcription units. It is unknown whether the albumin gene, if transcribed, would be represented in the cytoplasmic RNA of these cells.

The C2-Rev7 cell line, although derived from C2, can hardly be considered as a simple revertant to the same state as the parental FAO. While C2-Rev7 cells have regained the ability to transcribe the endogenous albumin gene at ~5% the rate of FAO cells, these cells contain an almost equal amount of mRNA as FAO cells. Likewise the rate of transcription of PepCK and PH genes is similar in all three cell lines but C2-Rev7 cells, like FAO cells, accumulate these mRNAs (Clayton *et al.*, 1985). The same applies to viral gene transcripts in C2-Rev7; they are synthesized at a normal rate and accumulate as well in C2-Rev7 as in FAO. Thus a major component in the 'reversion' of C2-Rev7 cells lies at the post-transcriptional level.

C2-Rev7 does exhibit one definite transcriptional change, however, as well as the described post-transcriptional changes. The albumin promoter in the adenovirus genome is used ~30% as well in C2-Rev7 cells as FAO cells, and is transcribed better than the endogenous albumin promoter in these cells. (This contrasts with no use of either endogenous or exogenous albumin promoters in C2 cells.) Thus C2-Rev7 cells in contrast to C2 would appear to have regained a *trans*-acting factor(s) that activates the exogenous albumin gene well. This factor by itself, however, can only poorly activate the endogenous gene. Possibly the different rate of transcription between the endogenous and exogenous gene in this cell line involves factors other than those which interact with albumin DNA sequences between the DNA segment present in the virus (-441 to +957) (Babiss *et al.*, 1986).

Thus the changes that occur among these cell types can affect both transcriptional and post-transcriptional machinery. It is far from clear that mutations and reversions in genomic DNA, rather than epigenetic changes occurred in the transition from FAO to C2 and then to C2-Rev7. Moreover, whatever changes occurred could have required multiple steps. It is nevertheless remarkable that these changes did affect, and in at least a partly reversible manner, both transcription and mRNA accumulation.

The use of recombinant adenoviruses should assist in determining whether the primary transcripts of the viral E2 gene as well as the PepCK and PH genes in C2 cells are correctly processed or whether they are lost before successful processing. It is possible that processing is normal but the defect lies in correctly transporting and/or stabilizing mRNAs in the C2 cell cytoplasm. This is an important area of mRNA metabolism about which almost nothing is known. It is certainly possible that a connection exists between successful transcription units in the nucleus and the structure that delivers mRNA successfully to the cyto-

plasm. For example it has been proposed that active genes might be associated with nuclear pores where their primary transcripts would, after correct processing enjoy advantages for effective transport to the cytoplasm (Blobel, 1985). In fact evidence has been presented for active transcription in localized sites in the periphery of the nucleus which could be associated with nuclear pores (Hutchison and Weintraub, 1985).

If the molecular basis for changes between the three cell types described could be determined perhaps progress could be made toward understanding the presently mysterious area of nuclear structure and mRNA transport or stabilization.

Materials and methods

Cell lines and viruses

The viruses used in this study (Figure 2), which are propagated in 293 cells (E1a and E1b containing cells), are recombinant adenoviruses in which the viral E1a gene and E1b promoter have been deleted and replaced by the rat albumin promoter (Babiss *et al.*, 1986; Friedman *et al.*, 1986). The alb454 virus contains 454 nucleotides from the left-end of wild-type virus including the E1a enhancer sequences but not the E1a cap site. This is fused to the rat albumin promoter region (−441 to +957) and the first two exons of the albumin gene fused within an intron to the E1b region, so that the 3' E1b exon and E1b poly(A) site allow the formation of a stable mRNA from the recombinant transcription unit.

The cell lines used in these studies are clonal descendants of line H4IIEC3 (Pitot *et al.*, 1964) derived from the Reuber H35 rat hepatoma (Reuber, 1961); and characterized by Deschatrette and Weiss (1974) and Deschatrette *et al.* (1980). All hepatoma cells were cultured in 10-cm Falcon Petri dishes in modified (Coon and Weiss, 1969) Ham F12 medium supplemented with 5% fetal calf serum. Cultures were grown at 37°C in a humidified incubator flushed with 7% CO₂. Cells were detached for transfer and harvested with a saline solution containing 0.05% trypsin and 0.02% EDTA.

Assays for gene expression

The preparation of viral and cellular nucleic acids has been described previously (Babiss *et al.*, 1986; Friedman *et al.*, 1986). Two assays for gene expression from the viral and cellular gene were used. Transcription from a region of DNA can be scored directly by allowing RNA chain elongation in isolated nuclei in the presence of labeled triphosphates (Derman *et al.*, 1981; Hofer and Darnell, 1981; Clayton and Darnell, 1983) and hybridizing the labeled nuclear RNA to excess DNA bound to nitrocellulose filters. The following cDNA clones were used as probes on the filters; PepCK (Yoo-Warren *et al.*, 1983); and rat PH (gift of S.L.C. Woo) and actin (Cleveland *et al.*, 1980). We have also utilized a series of probes to score the transcriptional activity of the viral genes. In order to score the transcription rate of the viral albumin promoter we spotted three subclones of the E1b gene (downstream of the promoter): 1B-110, a *Sau3A* fragment spanning 3320–3430 bp; 1B-656, a *Bgl*–*Sst*I fragment 3320–3040 bp of the adenovirus genome; and 1B-SB6, a 500-bp *Sst*–*Bam* fragment from a plasmid bearing 0–15.5 map units of adenovirus 5 (see Figure 3). Since 1B-110 and 1B-656 overlapped they were always used in different filter hybridization experiments. The E2 and E4 probes were previously described (Friedman *et al.*, 1986).

The production of stable mRNA from the viral albumin promoters was scored by its ability to protect from nuclease digestion a labeled RNA probe prepared from an Sp6 subclone of a portion of the E1b region containing an intron–exon junction (Friedman *et al.*, 1986). Electrophoresis and autoradiography were used to detect the protected RNA segments. Two discrete RNA bands resulted from molecules spliced at the sites in the E1b 22S mRNA (183 and 195 nucleotides), or the site that generates the E1b 13S mRNA (195 nucleotides). These regions are noted at the top of Figure 1.

The sP6 RNase protection assay for the 260 nucleotide E2 fragment was described by Hart *et al.* (1985). The probe used to score actin was from a human actin clone and rat actin protects an 86 nucleotide fragment (Enoch *et al.*, 1986). The E4 probe was an *Sma*I–*Bgl*II fragment between 84 and 93 map units and E4 RNA protects a 287 nucleotide labeled fragment.

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