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EXPRESSION OF HEPATO-SPECIFIC FUNCTIONS IN HEPATOMA CELL LINES

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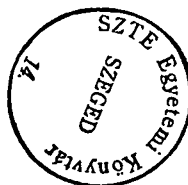

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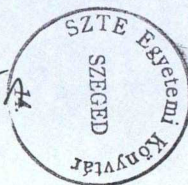

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Abbreviations

ADH - Alcohol dehydrogenase

ADH-L - Liver-specific alcohol dehydrogenase

Alb - Albumin

Ald - Aldolase

Ald-B - Liver type aldolase

AFP - Alpha-fetoprotein

AMP - Adenosine monophosphate

bp - base-pair

BUdR - 5-bromodeoxyuridine

C - Cytosine

cAMP - Cyclic AMP (Adenosine 3',5'-monophosphate)

cDNA - Complementary deoxyribonucleic acid

D - Dalton

Denhardt's solution 50x : 5g Ficoll, 5g polyvinylpyrolidone
5g bovine serum albumin, H O to 500 ml

Dex - Dexamethasone (9- fluoro-16 -methyl-prednisolone)

DNA - Deoxyribonuclei acid

DNase I - Deoxyribonuclease

EDTA - Ethylene diaminetraacetic acid

EMS - Ethyl-methansulfonate

FCS - Fetal calf serum

G - Guanine

gluc.rec. - Glucocorticoid receptor

Gr - Growth inhibition function

GRE - Glucocorticoid regulatory element

G3PDH - Glyceraldehyde 3-phosphate dehydrogenase

HAT - Hypoxanthine, Aminopterine, Thymidine

HPRT - Hypoxanthyne phosphoribosyl transferase

HS - Hypersensitive site

hsp - Heat shock protein

kb - Kilobase pairs

kD - Kilo dalton

LDH - Lactate dehydrogenase

ly - Lysis function

mRNA - Messenger ribonucleic acid

M.W. - Molecular weight

NP - Nonidet P

PBS - Phosphate buffered saline

PEG - Polyethylene glycol

PPO - 2,5-Diphenyloxazole

r - Growth resistance ; dexamethasone resistante

R- - Receptor deficient

Rwt - Wilde-type receptor

Rnt - Nuclear transfer-deficient receptor

Rnti - Increased nuclear-transfer receptor

Restriction endonucleases - Msp I; Hpa II; Eco RI; Hind III

rGH - Rat growth hormone

RNA - Ribonucleic acid

RNase - Ribonuclease

s - Growth sensitiviy

S-ADH - Stomach Alcohol dehydrogenase

SDS - Sodium dodecyl sulphate

ssc - Standard saline citrate: 150 mM sodium chloride, 15 mM
trisodium citrate pH 7.0

TAT - Thyrosine aminotransferase

TEB - electrophoresis buffer (0.09 M TRIS base, 0,09 M boric acid, 0.0025 M Na EDTA)

TK - Thimidine kinase

TRIS - Tris (hydroxymethyl) aminomethane

5' - Five primer end

5-Aza - 5-Azacytidine

5-mC - 5-Methylcitosine

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1. INTRODUCTION

Somatic cell hybridization is an important tool in the study of a number of biological phenomena like :

- the mechanisms of differentiation
- the regulation of gene expression of different genes
- mammalian cell transformation and tumorigenicity
- genetic complementation and gene mapping

Early studies were limited to biochemical analysis but more recently those can be combined with molecular genetic analysis Szpirer J. and Szpirer C. Cell 6:53-60 1975).

Such experiments have been carried out on a series of somatic cell hybrids between cells of a well-differentiated rat hepatoma and fibroblasts of diferent parental origin. It has been shown that fibroblasts contain factors which can extinguish the expression of liver-specific genes in some fibroblast-hepatoma hybrids.

Intra and interspecific hybrids were made involving hepatomas.

They may exhibit either an activation or a depression of liver-specific function which may suggest the presence of different factors within the cells, in different differentiation stages. There are several examples for depression of differentiated functions and others, where these functions are not extinguished.

Somatic cell hybrids derived from cell lines with different biochemical phenotypes presenting or not differentiated functions, should reveal whether there is a co-ordinate regulation

in the expression of different genes and by which mechanisms this regulation takes place.

In somatic cell hybrids the expression of differentiated function(s) can be:

1) maintained;

2) activated (new synthesis of a protein coded by a previously silent gene);

3) extinguished (disappearance of a previously expressed gene product);

The mechanisms of extinction or activation as well as the process of differentiation may involve trans-acting factors that interact with tissue-specific cis-acting control regions of the genome. The process of differentiation and dedifferentiation involves activation and repression of specific genes, these events give rise to the biochemical phenotypes of the differentiated, dedifferentiated cells. This specificity may be due partly to the trans-acting regulatory factors, and the study of such cells, as well as the study of cell hybrids between cells at different differentiation stages, suggests the presence of such factors within differentiated and dedifferentiated cells. Such tissue-specific trans-acting factors could control the expression of many transcribed genes in a particular tissue. One of the models for the action of these factors is that the expression of a single gene is controlled by different trans--acting factors present in different tissues.

These factors can be categorized into two groups : positive regulatory factors and negative regulatory factors.

Trans-acting control function is required for expression and

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inducibility of TAT (Tyrosine-aminotransferase), a liver-specific enzyme (Schmid W. et al, Proc.Natl.Acad.Sci.USA Vol.82:2866-2868 1985 ; Killary A.M. and Fournier R.E.K. Cell vol.38:523-534 1984). This function (a regulatory gene) has been assigned to chromosome 7, near the albino locus in mouse.

The TAT structural gene is present in homozygotes for the deletion on chromosome 7 near the albino locus but the enzyme level is dramatically reduced, on the other hand, is expressed normally in somatic cell hybrids between mouse liver cells homozygous for the deletion and rat hepatoma cells. In this situation, the absence of trans-acting factor/s, required for the normal expression of mouse TAT is complemented with the one present in rat hepatoma cells. A similar result was subsequently obtained for glucose-5-phosphatase, also a liver-specific enzyme (Schmid W. et al. Proc. Natl. Acad.Sci.USA vol.82:2866-2869 1985, Cori C. F. et al. Proc. Natl. Acad. Sci.USA vol.78:479-483 1981).

In the case of mouse alpha-fetoprotein /AFP/, the adult basal level of AFP mRNA is controlled by raf gene and the inducibility of AFP mRNA by the rif gene. It is probable that these genes are acting through trans-acting factors also (Olsson M. et al. Jour.Exp.Med. 145: 145:819-827 1971, Belayew A. et Tihghman S.M. Mol.Cell Biol. 4: 898-907 1982).

These changes in gene activities are generally referred to be epigenetic. It is unlikely that such changes in gene expression involve irreversible genetic changes, like a mutation or a chromosomal rearrangement. At least one exception exists of this theory - the differentiation of cells that synthesize immunoglo-

buline (Tonegawa S. Nature London 302:575 1983).

As mentioned before, highly specific protein (trans-acting factors)-DNA interactions are responsable for epigenetic changes.

One essential feature of these interactions is the post-synthetic chemical modification of bases in DNA. One of the most important DNA modification is the cytosine methylation, that occurs mainly in CpG dinucleotide in the DNA of higher organisms being envisaged as potential regulatory signal in eukaryotic gene expression (Doerfler W. Ann.Rev.Bioch.vol.52: 93-124 1983).

There are several evidences that the gene transcription is correlated with the absence of methylation at one or more sites in the promotor or sometimes other regions of the genes, in cells not expressing the given gene these sites were found to be methylated. The general hypothesis for the control of gene expression by methylation is that specific protein transcription factors recognize methylated or non methylated sequences and interact with RNA polymerases. It is proposed that the surrounding sequences are also essential for protein recognition. However it is still not clear whether methylation is a cause or an effect of gene inactivation, in either case, the pattern of methylation is inherited through DNA synthesis and cell division (Holliday R. Science vol.238:163-170 1987).

The importance of DNA methylation in gene expression is underlined by observations showing that 5-Azacytidine treatment of certain cells can activate silent genes by demethylation. 5-Azacytidine, a cytidine analogue is known to inhibit DNA methylation (Benvenisty N. et al. Biochem.24:5015-5019 1985).

Gene activity is probably subjected to multifactorial regulatory mechanisms, and DNA methylation constitutes only one important factor. Both chromatin and nucleosome structures are also important factors in these regulatory mechanisms, and there is evidence that these are correlated with the level of DNA methylation.

The above mentioned mechanisms with functions in gene regulation act principally at the transcriptional level through the levels of specific mRNA's.

The regulation of gene expression can also occur at the level of translation.

In the hepatoma cell lines, among the hepato-specific functions, there are examples of both.

The AFP and Alb, two major hepato-specific plasma proteins are regulated at the transcriptional level in rats (Jean-Louis N. et al. Jour.Biol.Chemy. vol.262 N26:12479-12487 1987). Contrary to these, in the case of TAT, a liver-specific enzyme, the data are very contradictory about the regulation of this gene. In the case of glucocorticoid induction of TAT it seems that the induction is regulated at a transcriptional level. However, in the case of induction by cAMP there is evidence for the transcriptional, (Hashimoto S. et al. Proc. Natl. Acad. Sci.USA vol.81 6637 - 6641 1984) translational control or for both (Noguchi T. et al. Jour. Biol. Chemistry vol.257, N5: 2286-2390 1982). For this and others reasons, the TAT expression is a very suitable model for the study of various aspects of regulation of gene expression in mammalian cells.

As was mentioned before, cell hybrids could be used in com-

plementation analysis.

Both inter and intraspecific hybrids can be used for these studies, in which it is possible to establish whether genetic defects in two mutant cell types, are in the same or in different genetic loci.

Glucocorticoids induce growth inhibition in Reuber hepatoma cell lines (Thompson E.B. et al. Gene Regulation by Steroid Hormons 1980; Venetianer A. et al. Cytogenet.Cell Genet. 28:280-283 1978), and this process requires functional intracellular receptor proteins and involves activation and nuclear transfer steps.

Pair-wise crosses between independent Gluc. resistant variants with recessive phenotype, would permit complementation analysis.

Positive results would allow an estimation of the minimum number of functional elements in the system.

In early 1960's, through chemical carcinogenesis the Reuber H35 hepatoma was obtained, which was adapted to grow "in vitro", giving rise to the hepatoma cell line H4IIEC3. All the hepatoma cell lines used in my study are descendants of this cell line. These descendants can be divided in two different groups : differentiated and dedifferentiated.

In our laboratory, from the dedifferentiated cell line H56 through the combination of mutagenic treatment and selection in the presence of Dex. (a synthetic glucocorticoid) a number of receptor less Dex. resistant variants were obtained.

On the other hand, from the differentiated cell line Faza 967 cultured in the presence of increasing concentrations of Dex.,

cell lines with reduced Dex. sensitivity were obtained but with normal glucocorticoid receptor number, as in the parental cell line. By culturing these Dex. resistant cell lines "in vitro", they maintained the hepato-specific functions (differentiated variants), some of them expressing hepato-specific plasma proteins such as AFP, not found in the parental cell line. Maintained in cell culture for a long time, they lose partially or totally these hepato-specific functions (partially dedifferentiated or totally dedifferentiated variants).

We also have cell lines phenotypically differents in terms of TAT expression.

	Basal TAT	Induced TAT
a)	++	+++++
b)	+	++
c)	++	+++

These variants are phenotypically different, being in different differentiation stages, expressing different hepato-specific functions. These variants offer a valuable model system for studing a whole range of liver-specific functions in the same cells and some of these functions e.g. TAT are influenced by glucocorticoids.

This model system give us the possibility to formulate the following questions :

- what are the phenotypes of cell hybrids between differentiated and dedifferentiated hepatoma cell lines?
- at what level is the regulation of same hepato-specific functions (Alb, AFP, TAT)?
- what are the methylation patterns of the 5'region of AFP

and Alb in parental and hybrid cell lines?

- what is the possibility of complementation of Dex resistant hepatoma cell lines?

In answering these questions, we can give our contribution to the understanding of the regulation of expression of liver-specific functions in hepatoma cell lines.

1.1 Alpha-fetoprotein and Albumin

Alpha-fetoprotein (AFP) is one of the most important plasma protein during foetal life, and drastically decrease in the serum of adult animals. On the contrary, Alb is one of most important plasma proteins during adult life, and it's concentration increases from low levels early in foetal development to high levels in postnatal life (A.Venetianer et al. Differentiation 31:148-156 1986 ;Jose M.Sala-Trepat et al. Proc.Natl. Acad. Sci. USA Vol.76:695-699 1979).

These plasma proteins are considered markers of hepatic differentiation. Changes in the activity of these two genes are caused by stimuli that also alter the hepatic differentiation state. The AFP synthesis is also influenced by carcinogenesis and regenerative processes of the liver in adult mammals,re-expression at high levels of AFP appears in hepatocarcinomas and regenerative processes (Belanger L. et al. Adv.Enzyme Regu. 21: 73-99 1983; Nechaud B. and Uriel J. Intl.J.Cancer 8:71-80 1971).

AFP and Alb are mainly secreted by the liver. AFP is similar in structure and exhibits certain physiochemical and functional similarities to serum Alb, with considerable amino acid homolo-

gy. Serum Alb is 66000 D and AFP 72000 D, both are single polypeptid chains, with similar functions probably as ligand-binding proteins, suited in some undefined fashion to the foetal circulation (Joseph L. et al.Carcinogenesis vol.8:241-246 1987).

The AFP and Alb are encoded by structural, closely related genes, each one is 20 kb pairs long and is split in 15 exons by 14 intervening sequences, Fig.1 A, 2 A; they derived from duplication of a commom ancestral gene and organized in tandem (5'Alb, AFP3') with the same polarity of transcription in mouse, human and rat(Jean-Louis Nahon Jour. Biol. Chemy. vol.262 N26 12479-12487 1987).

The phenotypic properties of the AFP and Alb gene system provides a model to analyse developmental gene controls superimposed on tissue-specific control in normal cells and in hepatoma cells.

Jose M. Sala-Trepat and collaborators (Proc.Natl.Acad.Sci.USA vol.76 695-699 1979) excludes the possibility that high levels of Alb and AFP during hepatic carcinogenesis could be explained by gene amplification. He forwards the hypothesis that a transcriptional activation or a post-transcriptional stabilization of AFP mRNA could be responsable for the increase of AFP levels during hepatic carcinogenesis.

Andras Gal et al. (Differentiation 29:238-242 1985) start from the fact that different changes in DNA as :rearrangement, base substitution or base modification, could cause selective gene activation or repression during the organisms development as happens in several others genes. On the results of their work the hypothesis that rearrangements are the basis of

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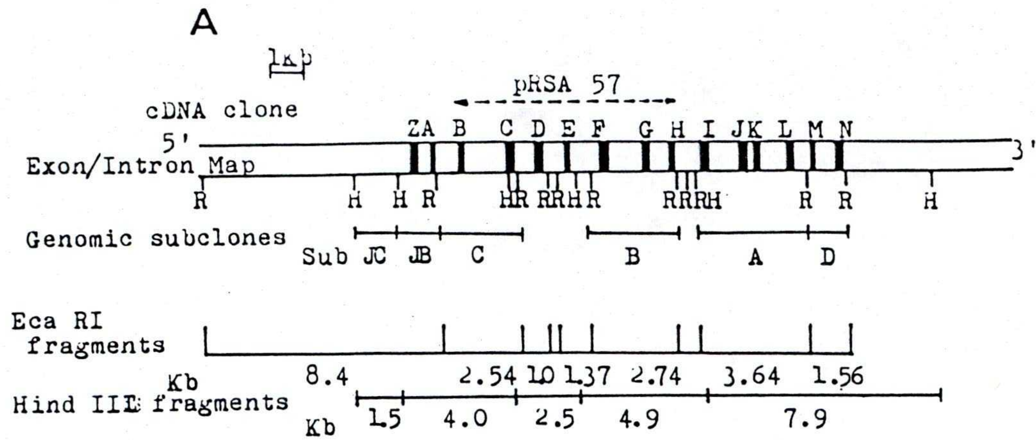

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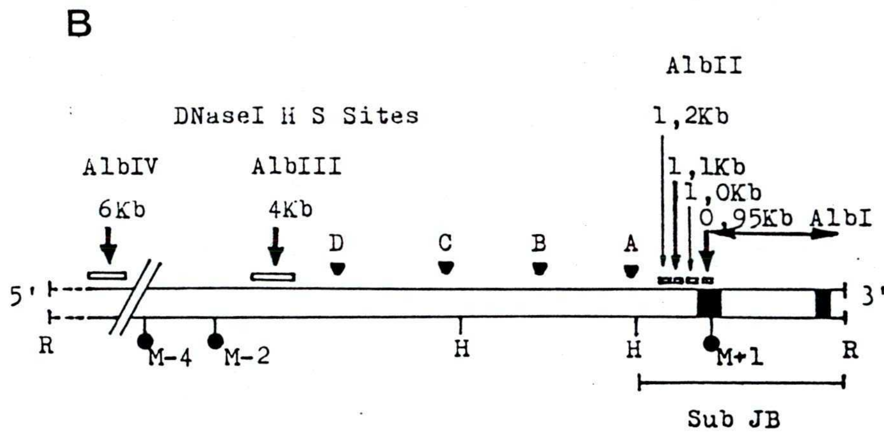


Fig. 1 A. Physical map of the rat serum albumin gene. Black vertical boxes denote exons and white boxes denote introns, Hind III (H), EcoRI (R), from (Gal A. et al. Differe. 29:238-242 1985).

B. DNase I HS and methylation sites upstream from the rat Alb gene. The major DNase I HS sites are noted Alb I, Alb II, Alb III, Alb IV, the minor DNase I HS sites are noted A, B, C, D; the methylation sites are M+1, M-2, M-4, from Tratner J. et al. Mol. Cell Biol. 7:1856-1864 1987).

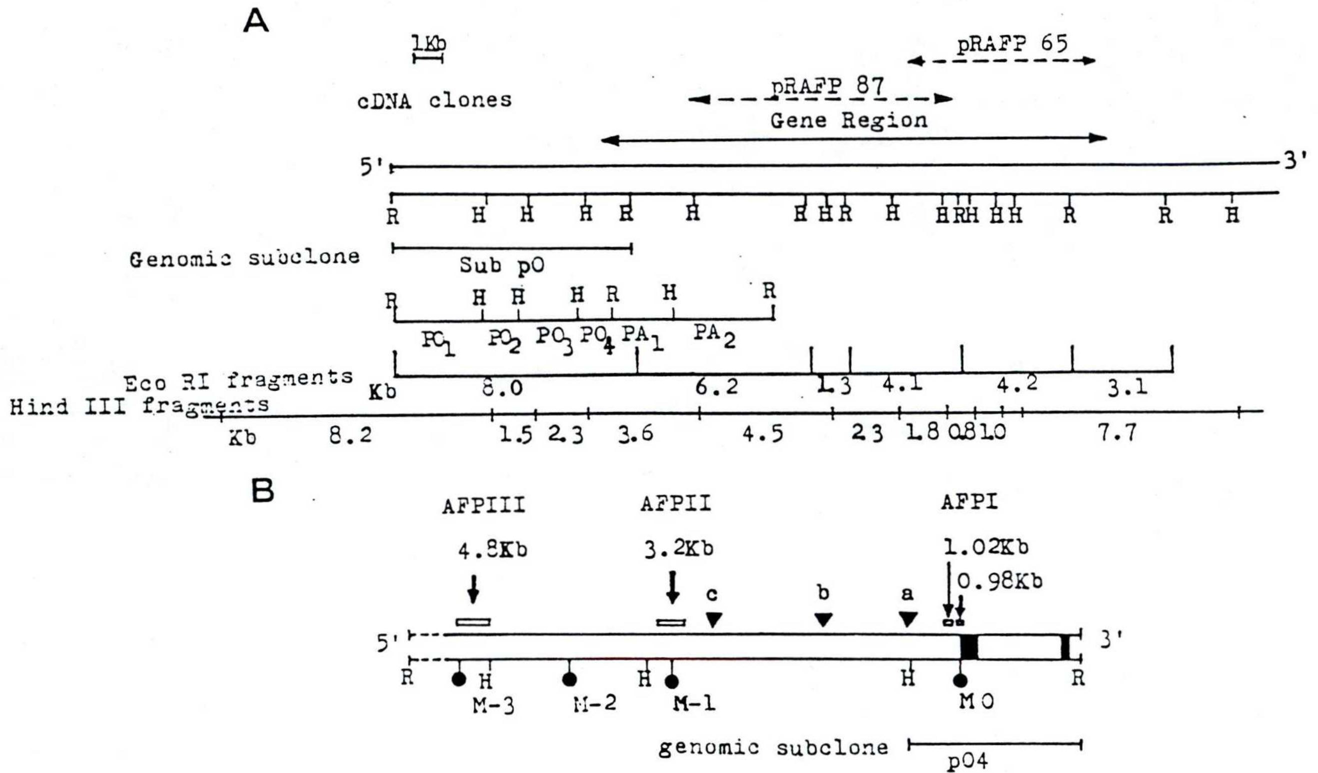


Fig. 2 A . Physical map of the rat AFP gene EcoRI and Hind III sites. EcoRI (R), Hind III (H), cDNA and genomic clones.

B . DNase I HS and methylation sites upstream from the rat AFP gene. The major DNase I HS sites are noted AFP I, AFP II, AFP III, the minor with a, b, c; methylation sites are M 0, M-1, M-2, M-3, from Tratner J. et al. Mol.Cell Biol. 7:1856-1864 1987.

transcriptional changes during development çannot be considered true in rat hepatomas, unless these recombinations are small (0,1 kb). Their work provides data about this gene variation in different rat lineages, and especially the existence of two different structural variants in Alb and AFP genes. The same author in two others works (Mol. Gen. Genet. 195: 153-158 1984, Bioch. Genet.vol.23 N3/4 :257-265 1985) shows the existence of two structural variants of this gene in mouse. However, there is no size differences of AFP mRNA between the two types above mentioned and therefore this polymorphism does not have repercussion at the phenotypic level.

Jean-Louis Nahon et al. (Proc. Natl. Acad. Sci. USA vol.81 : 5031-5035 1984) forwards the possibility of correlation between cromatid structure changes and transcription of Alb and AFP gene. They conclude that there is no evidence to sustain that alteration in the cromatid structure of Alb and AFP gene could be responsible for the change of gene activity during hepatocytes differentiation. However, there is different cromatid conformation, meaning different DNase I sensitivity of these genes, in different tissues.

There is still a possibility of other differences in DNaseI sensitivity that were not detected, in special in 5' region of these genes, which has been lately associated in several studies with active gene transcription.

In the recent years, a lot of work has been done searching for the correlation between DNA methylation and gene expression.

One of these works, by Marie-Odile Ott et al. (Cell vol. 30: 825-833 1982), conclude that submethylation of transcript unit

of Alb gene is not necessary to the Albumin gene expression. The demethylation of a specific methylation site named M 1 in 5' region is necessary but not sufficient for stable gene expression.

Another work about the DNA methylation of these genes was carried out by Michele Vedel and co-workers (Nuc. Acid. Rese. vol.11 N 13: 4335-4354 1983). They found a correlation between hypomethylation and the expression of Alb and AFP genes in adult rat liver and in neoplasic cell lines of hepatic origine. No correlation between hypomethylation and the expression of Alb and AFP genes during normal development of the organism was found.

A complete work about methylation and DNase I sensitivity in the 5' region of Alb and AFP genes, was performed in France in "Centre National de la Recherche Cientifique" Gif-sur-Yvette by Tratner I. and co-workers (Mol. Cell Biol. vol.7, N5: 1856-1864 1987). As conclusions of their work, they found that the gene expression of Alb and AFP is well correlated with the existence of DNase I hypersensitivite sites and with specific demethylation of MspI sites in 5' region of the genes.

Two MspI sites M-2, M 0 in 5' region of AFP gene and one M+1 in 5' region of Alb gene are demethylated when these genes are actively transcribed. DNase I hypersensitivite sites AFP I, Alb I and Alb II are detected only when these genes are actively transcribed.

Of particular interest, is the close localization of some Msp I and DNase I HS sites, Fig. 1 B, 2 B.

It is very likely that hypomethylation and hypersensitivity

to Dnase I interact to define sequences involved in interaction with specific regulatory proteins. The synthesis of AFP and Alb are mainly regulated at transcriptional level in hepatomas and also during rodent development (Nahon J. L. et al. Proc. Natl. Acad.Sci.USA 81: 5031-5035 1982).

The previous work is very important for us, because we will examine the same methylation sites in the 5' region of these two genes.

1.2 Tyrosine aminotransferase

The L-tyrosine-2-oxoglutarate aminotransferase (EC 2.6.1.5.) (TAT) is a aminotransferase, the first and rat-limiting enzyme in tyrosine degradation, characteristic of the liver and hepatoma cell lines and almost exclusively located in this organ.

It is an enzyme, whose activity appears first at birth. Beginning at the second hour after birth, TAT activity increases strikingly, 12 hours after birth reaches a maximum, at least twice that of adult levels. The enzyme activity gradually drops so that it returns to the basal levels, values observed in livers from adult rats, at 2 days after birth (Sereni F. et al. Jour. Biol. Chem. 234, N3: 609-612 1959). It has been proposed that during the time of transition from late fetus to newborn, that the increasing of plasma glucacon and hydrocortisone levels and falling insulin levels are hormonal signals for the synthesis of TAT (Ghisalberti A.V. et al. Biochem. 190:685, 1980).

The native form of TAT is a dimer comprised of subunits of 52,000 D, designated form I. Two other forms, resulting from a

limited proteolysis of the form I, are commonly isolated from rat liver.

The TAT gene is a single copy gene about 11 kb with 11 introns, the mRNA is 2400 bp long (Fig. 3). Only about 1400 nucleotides of mRNA are necessary to encode tha 52,000 D TAT protein (Shinomiya T. et al. Proc. Natl. Acad. Sci. USA vol.81: 1346-1350 1984).

The expression of TAT is tissue-specific and developmentally regulated like the AFP and Alb genes.

Steroid hormones as Dex. induces the synthesis of TAT in rat hepatoma tissue culture cells (Baxter J.D. et al.Proc.Natl.Acad. Sci. USA 65 : 709-715 1970). The induction of liver TAT activity in vivo is correlated with cytosolic glucocorticoid receptor levels (Tanvis A.S. et al.Biochim.Biophys.Acta 886 :162-168 1986).

The cyclic AMP and active analogous such as N6,02-dibutyryladenosine 3',5' cyclic monophosphate (Bt2-cAMP) increase the TAT enzyme activity, however, there is some doubt about the way of action.

Wicks W.D. concluded that the induction by cAMP was exerted at a post-transcriptional level (Wicks W.D. Adv.Cyclic Nucleotid Res. 4:335-438 1974). Other groups, have more recently shown, that cAMP administration resulted in a rapid increase in TAT mRNA (Hashimoto S. et al. Proc. Natl. Acad. Sci. USA vol. 81: 6637-6641 1984).

The results presented by Noguchi T. suggest that tyrosine aminotransferase synthesis is regulated in two ways, after intraperitoneal injection of Bt2cAMP (Noguchi T. et al. Jour. of Biol.Chem. vol.257:2386-2390 1982).

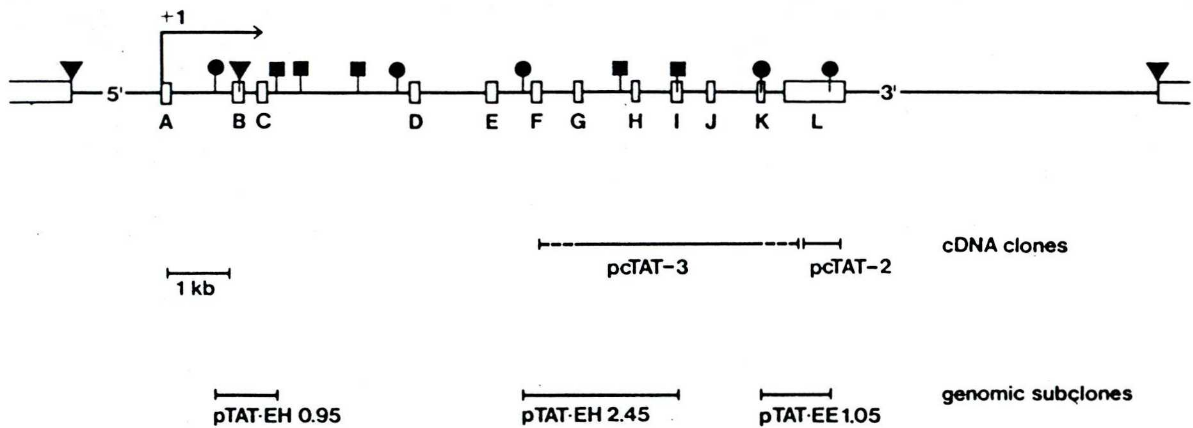


Fig. 3 . Physical map of the rat TAT gene, cDNA clones and genomic clones.

Exons are shown by open boxes, from Hashimoto S. et al. Proc. Natl. Acad. Sci. USA 81:6637-6641 1984.

Meyer R. et al. (Bioche.Biophy.Acta 886: 143-151 1986) demonstrated a synergistic interaction of hydrocortisone and Bt2-cAMP.

One important work about the regulation of expression of the TAT gene was carried out by Becker P.B. and co-workers (Cell 51: 435-443 1987). They have shown the existence of a specific DNase I hypersensitive (HS) sites and the demethylated stage of specific sites in the 5'flanking region of the TAT gene in the TAT-expressing cells, but not in the nonexpressing cells. One important result was that the CpG dinucleotides within the regions of protein-DNA interaction in the 5'end of the TAT gene are heavily methylated in the nonexpressing cell lines and tissues.

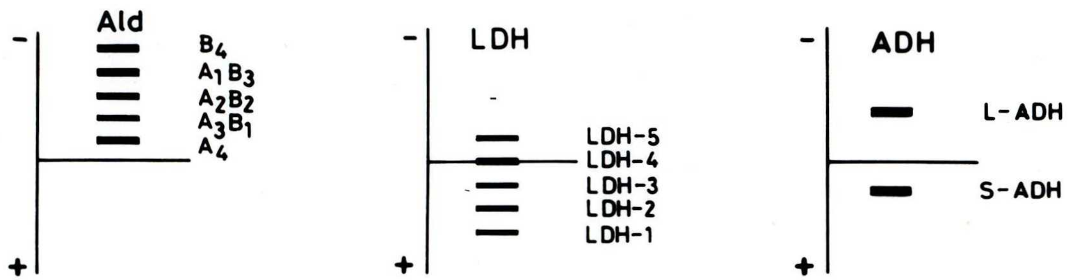
1.3 Aldolase

The Aldolase enzyme (Fructose 1-6 biphosphate aldolase E.C.4. 1.2.13.Ald.) catalyses the cleaving of the fructose 1-6 biphosphate into the dihydroxyacetone phosphate and glyceraldehyde 3-phosphate.

Three different (subunit) types of aldolase isozyme have been found : type A, type B, type C. Each isozyme contains four subunits. Two types of subunits by association could give five forms, including three hybrid forms, these are detectable by zone electrophoresis Fig.4 A, and three types of subunits can give fifteen forms.

The homotetramer of subunit type A called Ald-A is the muscle type, being the principal form in the muscle cells, blood cells and in fibroblasts but is also expressed in kidney, brain and

A



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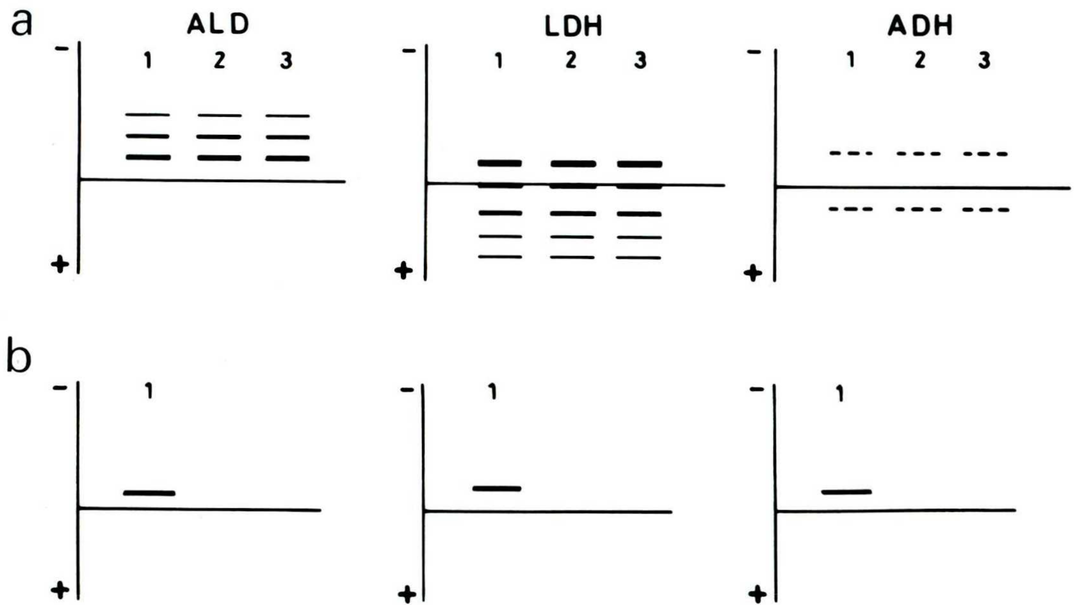


Fig. 4 A . Schematic reproduction of zymograms of Ald, LDH, ADH, from hepatic extract, the possible forms.

B . Schematic reproduction of zymograms of Ald, LDH, ADH, from parental cell lines. a) Fα6 cell line 1.62, 2.76, 3-92 days efter fusion, b) H56 cell line 1.52 days before the fusion.

liver, together with other forms.

The homotetramer of subunit B called Ald-B, is the liver type and is expressed in the liver and small intestine.

The homotetramer of subunit C called Ald-C is selectively expressed in the brain. Very low levels have also been found in others tissues. Functionally it is similar to Ald-A (Penhoet B. et al. Proc.Natl.Acad.Sci.USA 56: 1275-1282 1966).

All available data exclude the possibility of generating the different iozyme forms from a single gene and imply the presen-ce of three different genes.

The homology between aldolase subunit types indicates that they probably origineted from a common ancestral gene (Paolella G. et al.Eur.J.Biochem. 156:229-235 1986).

The Ald-B gene comprises about 14 kb of DNA, and nine exons (Tsutsum K et al. Isozimmes Current Topics in Biological and Medical Research vol.14: 177-193, Alan. R. Liss, Inc.1987).

The expression of these subunits in various animal tissues is tissue-specific and developmentally controlled. The catalytic properties of Ald A and B can be correlated with physiological functions in the tissues in which they were found.

The mechanism by which this tissue-specific regulation is made is not well known, however is basically determined by the mRNA concentration, controlled at the transcriptional level.

In the differentiated hepatoma cells Fig.4 A, Ald-A and B and heterotetramers are visualized by electrophoresis.

1.4 Alcohol dehydrogenase

The Alcohol dehydrogenase (alcohol;NAD+ oxidoreductase, ADH, E.C.1.1.1.1.) catalyses the conversion of ethanol to acetaldehyde, the first reaction in the two-step oxidation of ethanol via acetaldehyde to acetate and this being the major pathway of ethanol oxidation.

The first step is the rate limiting step in the pathway of ethanol metabolism and the level of ADH in the liver is the major determinant of the rate of ethanol elimination in rats.

The level of liver ADH in rat increases through development. In the eighteenth day of gestation it is detectable in the rat fetus and increases to 25% of the adult level at birth. The adult level is reached 18 days later (Niels C. R. R. et al. Biochem. J. 103: 623-626 1967).

The ADH activity in female rats is higher then in male rats and Crabb D.W. demonstrated that this sexual difference in ADH activity in rats is under hypothalamic control (Crabb D.W. et al. Life Sci. 37:2381-2387 1985).

The ADH is a dimer with molecular weight of 80,000 D and the monomer of the rat liver ADH have a molecular weight very close to 40,000 D.

The differentiated hepatoma cells reveal the presence of two bands of activity; the cathodal liver isozyme L-ADH and the anodal stomach S-ADH, see Fig. 4 A, (Deschatrette J. et al. Biochem. 56: 1603-1611 1974).

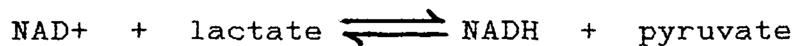
Another tissue-specific ADH isozyme has been identified recently, a protein from rat testis that catalyses the oxidation

of ethanol in the presence of NAD+ (Chiao Y-B. et al. Alcohol 21: 9-16 1986).

The rat ocular tissues shows two anodal isozymes of ADH, one of them the ADH1, corresponding to more then 95% of all ADH activity in the eye (Julia P. et al. Exp. Eye Res. 42 (4): 305-314 1986).

1.5 Lactate dehydrogenase

L-lactate dehydrogenase (L-lactate NAD+ oxydoreductase E.C. 1.1.1.27, LDH) plays a critical role in glucolytic metabolism and carries out a straightforward reaction :


NAD+ + lactate ⇌ NADH + pyruvate

The LDH isozymes are not hepato-specific, however they are found in the liver, see Fig.4 A.

The enzyme exists as isozyme. These isozymes are tetramers generated by largely random association of LDH subunits within cells among mammals and birds (Markert C.L. Science 14:1329-1330 1963), forming a complex gene family. Two different subunits can generate five tetrameric isozymes : A4, A1B3, A3B1, B4, (Fig.4 A) and three can generate fifteen isozymes.

Each subunit has a molecular weight of about 35,000 D in all vertebrates. The relative abundance of the isozymes reflects the relative activities of the correspondings genes (Nadal-Giard B. Proc. Natl. Acad. Sci. USA 73 : 3618-3622 1976).

The subunit A or LDH-A encoded by the Ldh-1 gene is the predominant isozyme of the white skeletal muscle.

The subunit B or LDH-B encoded by Ldh-2 gene, is the predominant isozyme of the cardiac muscle.

In mammals and birds a third LDH gene was identified and codes a subunit called C. This isozyme called L4 or LDH-X exhibits a extremely restricted cellular expression, being active only in the primary spermatocytes.

One hypothesis for the evolution of these three genes is that LDH-A and LDH-B genes arose by a polyploidization and LDH-C arose from LDH-B by a regional duplication (Markert C.L. et al. Science vol.189:102-104 1975).

The existence of two other regulatory genes has been proposed in mouse. The Ldr-1 regulatory gene controls the expression of the LDH-B gene in erythrocytes (Shows T.B. et al. Proc.Natl. Acad.Sci.USA 61:574-581 1968). The other, Ldh-2 regulatory gene controls the level of LDH-B subunits in mouse liver (Khlebadrava T.M. et al. Biochem.Genet. 18:1027-1039 1980).

There has also been a regulatory gene reported in the rat.

1.6 Glucocorticoid hormone action

Glucocorticoids exert many of their physiological effects by modulating the expression of specific genes mainly at the transcriptional level. These effects are different in various cells, both in vivo and in vitro.

Some of the observed effects in cells are : inhibition of nutrients transport, decreased activities of polyamine synthesizing enzymes, inhibition of RNA and protein synthesis, influence the proliferation of specific target cells, eventually

Fig. 5 . The principal steps of the steroid hormone action. 1. enter the cell; 2. binding to the receptor; 3. activation or transformation; 4. enter the nucleus; 5. binding to specific DNA sites. S. steroid hormone; R. receptor; H. heat shock protein.

lysis of lymphoid cells and activates expression of the differentiated phenotype (Gehring U. Mol.Cell Endoc. 48: 89-96 1986).

The glucocorticoids, like other steroid hormones, act by binding to specific intracellular receptors, traverse the plasma membrane probably via protein carrier rather then by simple diffusion (Yamamoto K.R. Ann.Rev.Genet. 19:209-252 1985). The intracellular distribution of the receptor has been debated .

Initial studies proposed that the free receptors are cytoplasmatic, but recent investigation suggested that the nonliganded receptor reside predominantly in the nucleus.

Before the hormone binding, the free glucocorticoid receptor seems to exist in a complex with the 90 kD heat shock protein (hsp).

Teitz T. et al. (Proc. Natl. Acad. Sci.USA 84:8801-8804 1987) showed that in the molybdate-stabilised L-cell cytosol the glucocorticoid receptor in an untransformed state exists in a complex with the 90 kD hsp, and this hsp dissociates from the receptor during temperature-mediated transformation.

Whatever the predominant location of the free receptors is, these have a low affinity for DNA and their dissociation rates are rapid relative to those of hormone-receptor complexes. The glucocorticoid hormone binds to the cytoplasmatic receptor, leading rapidly to a conversion of the whole receptor population to the hormone bound state.

The glucocorticoid receptor complex passes through a activation or transformation process and this process is temperature dependent in whole cells. The transformation

increases the affinity of the receptor for binding sites in the cell nucleus.

After the nuclear transfer the glucocorticoid receptor-complex binds to the chromatin. This complex recognizes specific sequences, the glucocorticoid regulatory elements GRE, and affects the transcription of these genes. These GRE are hormone--dependent enhancers, whose activity appears to be fully dependent on the occupancy by the glucocorticoid receptor-hormone complex. A partially polindromic 15 bp element is sufficient to mediate the glucocorticoid action (Strahle V.et al. EMBO Jour. vol.7: 3389-3395 1988). These elements are located in the 5' flanking region of the genes, 50 bp from the transcriptional inhibition sites in the case of chicken lysozyme gene (Renkawitz R. et al.Cell 33: 503-510 1984) and more than 2,600 bp in the case of rabbit uteroglobin gene (Cato A. et al. Embo.Jour. 3: 2771- 2778 1984) or in the first exon in the case of human growth hormone (hGH)(Slater E.P. et al. Mol. Cell Biol. 5: 2984-2992 1985).

1.7 Glucocorticoid hormone receptor

A consequence of the chemical simplicity of steroid hormone is that, they themselves can neither encode nor decode the varied and complex genetic programme that they now simbolize. For this they need steroid receptors with sufficient chemical complexity to interprete a different sort of code, one that specifies the genetic changes comprising a hormone response.

The receptors are intracellular proteins, soluble in cell

extracts. Jacobson M. (unpublished data), indicates that the receptor gene encompasses more then 45 kb and contains many introns.

A comparison of the human and rat gluc. rec., the estrogene receptor and the v-erb-A oncogen product of avian erytroblastosis virus shows striking homology, essentially with the 66 amino acid DNA binding domain. Suggesting that these genes are members of a large gene family derived from a common primodial regulatory gene (Weinberg C. et al.Nature vol.318:670-672 1985).

Two types of glucocorticoid receptor mRNA's were identified in cells of different species and organs, a major mRNA about 7 kb and a minor of approximately 5 kb. These transcripts differ in the lengths of their untranslated 3' sequences corresponding to alternative polyadenylation sites (Gehring U. et al. Mol.Cell Endoc. 48: 89-96 1986).

The wild type receptor /Rwt/ is about 95,000 D and consists of 795 amino acids, the increased nuclear transfer type /Rnti/ have a M.W. of only 40,000 D and corresponds to a 5,5 kb mRNA from which 5' sequences are missing (Gehring U. and Hotz A. Biochemistry 22: 4013-4018 1983).

Kalinyak J.E. and co-workers (Jour.Biol.Chem. 262:10441-10444 1987) provide evidence for the regulation of gluc.rec. level by glucocorticoids.

The model for the glucocorticoid receptor proposes three functionally distinct domains which are linearly arranged along the receptor polypeptid :

- the hormone binding domain in the carboxy terminal position
- the DNA binding domain in the middle, this domain makes

possible the receptor interaction with nuclear acceptor sites or DNA.

- the modulator domain in the amino terminal of the molecule and this domain modulates the DNA binding and limits the receptor affinity for nonspecific DNA. It may also function by interacting with the transcription machinary or by affecting the structure of chromatin (Gehring U. Mol.Cell Endoc.48:.89-96 1986) Fig. 6.

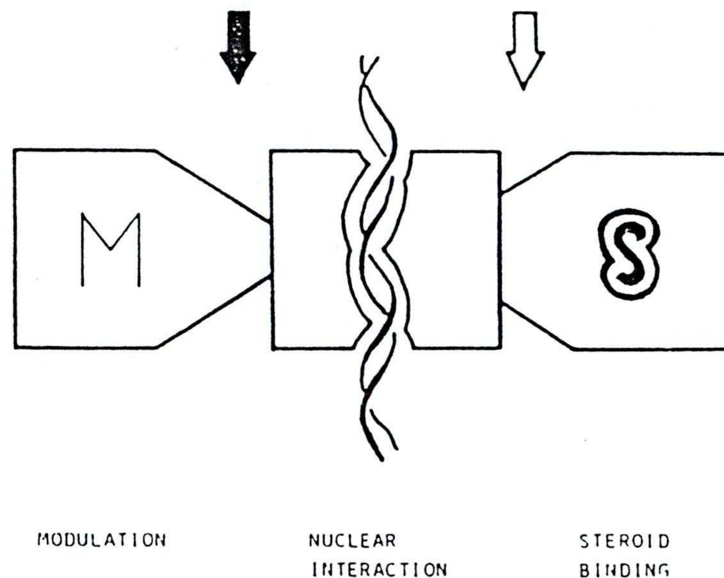


Fig. 6. Domain model of the wild-type receptor; from Gehring U. Mol.Cell Endoc. 48: 89-96 1986

Most of the glucocorticoid resistant lymphoid clones isolated reflect alteration either due to defects in receptor quantity or quality. The most predominant is the receptor deficient type R-. A second type is the nuclear transfer deficient receptor /Rnt-/, this is defective in the nuclear binding and have a decreased affinity for general DNA. Another type is the Rnti with increased binding to chromatin and abnormally high affinity for

DNA (Yamamoto K.R. et al. Recent Prog. Horm.Res. 32: 3-32 1976). Some different specific glucocorticoid resistant clones have been also characterized. An activation labile, extremely instable during attempted activation with rapid loss of hormone binding activity (Harmon J.M.et al. J.Ster.Biochem.21: 227-236 1984). A glucocorticoid induced resistance, was shown to be correlated with a reversible down regulation of receptor level by the hormone (Danielse M. and Stallcup M.R. Mol.Cell.Biol. 4:449-453 1984). A few examples of steroid-insensitive cell lines possessing apparently normal receptors have been published (Darbre P.D. et al. Cell vol.51:521-528 1987).

A few complementation analyses of differnt glucocorticoid resistant cell lines were attempted.

The isolated hybrid clones by Yuh Y-S. and Thompson E.B. (Som. Cell and Mol.Genet. vol.13:33-46 1987), from human lymphoblastoid R+ lysis function defective /ly/ and R- ly+ parental cell lines, were shown to be Dex sensitive. Complementation was also obtained between spontaneous mouse lymphoma cells with functional gluc. rec. and receptor deficient murine thymoma cells (Gasson J.C. and Bourgeos S. Jour. Cell biol. 96:409-414 1983); and between cell lines derived from fibroblasts by Gal A. and Venetianer A. (Cytog. Cell Genet. 35:75-77 1983).

1.8 DNA methylation.

One of the most important DNA modifications is the DNA methylation. The modified bases in the DNA of prokaryotes is the N6-methyladenine (6-mA) and 5-methylcytosine (5-mC). DNA of higher

eukaryotes contain 5-mC, as the modified base and is about 2 to 8 mol% in mammals.

In average, 70% of all CG sequences are methylated in animal cell DNA.

The pattern of DNA methylation is inheritable and is maintained by the action of DNA methyltransferase in a early post-replicative step.

Two different DNA methyltransferases are now known. The maintenance DNA methyltransferase, which acts on hemimethylated DNA, methylate bases in the newly synthetized DNA strand in an antiparalell mirror-like fashion and the novo DNA-methyltransferase acting in the DNA, not previously methylated and imposes a sequence specific pattern of DNA methylation.

One important question is how the action of the novo methyltransferase is regulated. It is conceivable that the access of DNA methyltransferase to specific DNA sites is regulated by chromatin proteins (Creusot F. Nuc.Acid.Res. 9:5359-5381 1981).

Several studies were published about the DNA methylation and the results have given evidence of the implication of DNA mathylation in a number of biological processes.

The DNA methylation may act according to Doerfler W. (Ann. Rev.Biochem.52:93-124 1983) in the following way:

- DNA protein interaction, assuming positive or negative regulatory signal or by specific and localised structural alteration of DNA
- modulation of protein-DNA interaction and as a long-term signal
- protection of DNA against restriction endonucleases

- enhancment of mutation and recombination
- DNA replication influence, virus latency and differentiation

A number of sensitive techniques have been developed for the quantification and detection of 5-mC in a given sequence. One of the most useful techniques is based in the use of restriction endonucleases such as the MspI, HpaII isoschisomers. The use of restriction endonucleases yields a somewhat limited answer, because only a few isoschisomeric pairs are available (Bird P.A. Jour.Mol.Biol. 118:49-60 1974).

The 5-Aza can be incorporeted into replicating DNA but cannot be methylated and in this state inhibits the activity of DNA methyltransferase perhaps by irreversibly binding these enzymes.

Inverse correlation between the degree of DNA methylation of certain genes and the extent to which these genes are expressed has been estabilished.

A decrease in DNA methylation is observed in 5-Aza treated cells. 0,3% substitution of cytidine by 5-Aza is sufficient to inactivate more than 95% of the enzyme in the cell (Creusot F.et al. Jour.Biol.Chem. 257:2041-2048 1982).

The 5-Aza can be used to activate previously inactivate genes (Bevenisty N. et al. Biochem. 24:5015-5019 1985). The human HPRT gene from a structurally normal inactive human X chromosome retained in a mouse-human somatic cell hybrid is expressed after 5-Aza treatment (Peter A.J. et al.Proc.Natl.Acad.Sci.USA vol.79: 1215-1219 1982).

The effect of 5-Aza is totally reversible and does not exert significant mutagenic action in eukaryotic cells.

2. MATERIALS AND METHODS

The following techniques can be divided in three groups : the first, somatic cell techniques; the second, biochemical techniques for the biochemical characterization of the cell lines; the third, molecular techniques used for the characterization at molecular level of the same cell lines.

2.1 Somatic cell techniques

2.1.1 Cell lines

All hepatoma cell lines used were derived from the H4IIEC3 line adapted to growth in vitro from the Reuber H35 hepatoma (Pitot et al Nat.Cancer Inst.Monogr.12:229-246 1964).

The filiation of the cell lines used in this study is summarized in Fig. 7.

A part of the dedifferentiated cell lines used, have a common origin H56 cell line, isolated from the BUdR (5-bromodeoxyudine)resistant H5 cell line. The H56 cell line was isolated and characterized by Deschatrette J. and Weiss M.C. (Cell vol.19: 1043-1051 1980; Biochemie 56:1603-1611 1974) and by Venetianer A. et al.(Cytogenet. Cell Genet. 28: 280-283 1980).The H56 cell line is dex-sensitive /s/, glucocorticoid receptor positive /R+/, TK-, AFP-, Alb-, TAT-, ADH- , LDH-,Ald-.

The H56 cell line was mutagenised in a single step with 400 µg/ml EMS during 20 hours with a survival rate of 30%, giving rise the S-H56 cells. They were kept 3 months in cell culture in

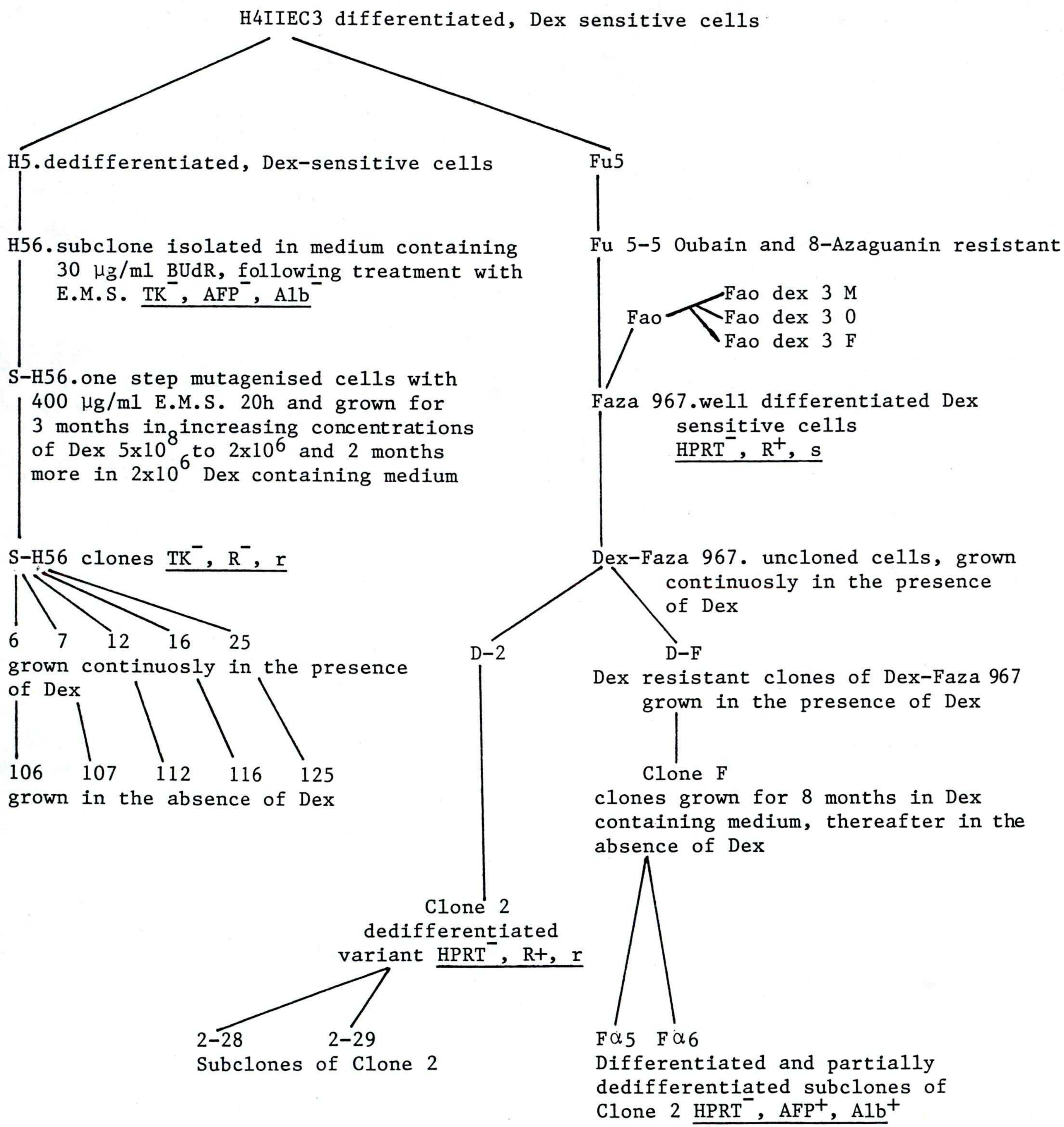


Fig. 7 . Filiation of cell lines used in the study.

Dex. presence, starting with $5x10^{-8}$ Dex. concentration, rising every 3 weeks in such a way to obtain 3 months later the concentration of $2x10^{-6}$ Dex./ml. It was kept during 2 months in culture in presence of $2x10^{-6}$ Dex./ml, and after that was cloned and each clone divided in two parts : one of them was kept in Dex. presence, designated as S-H56 (6,7,12,25), and the other in Dex. absence, designated as S-H56(106,107,112,125). These clones are TK-, Dex. resistant /r/, glucocorticoid receptor minus /R-/, and dedifferentiated like the H56 cell line.

The remainder cell lines are derivative from Fu5 (J.Deschatrette et M.C.Weiss Biochimie 56:1603-1611 1974). The Ouabain resistant cell line designated Fao, was isolated from the 8-azaguanin resistant hepatoma line (HPRT-), designated as Faza 967, a differentiated, Dex.-sensitive (growth sensitivity) cell line with numerous functions characteristic of adult liver hepatocytes e.g., the production and secretion of serum albumin, Alb+, the basal activity of tyrosine aminotransferase TAT+ and the inducibility of TAT by glucocorticoids, the presence of liver-specfic isozymes of aldolase (Ald-B+), alcohol-dehydrogenase (ADH-L+).

The Fao dx3 M cell line was isolated from Fao cell line cultured in the presence of increasing concentration of Dexamethasone, from 10^{-7} to $2x10^{-6}$ M, and maintained continuously in the presence of $2x10^{-6}$ M Dex. The Fao dx3 F and Fao dx3 O were derived from the Fao dx3 M, all with different TAT levels.

On the other hand a Dex-Faza 967 cell line was obtained by growing Faza 967 cells in the presence of increase concentrations of Dex. from $1x10^{-7}$ to $2x10^{-6}$ for 4 months (A.

Venetianer et al Cytogenet. Cell Genet. vol.28:280-283 1980).

By cloning the Dex-resistant Faza 967 cells in medium containing $2x10^{-6}$ M Dex, different variants, clone designated D-2 dedifferentiated and D-F the differentiated and partially dedifferentiated variants, were obtained. These clones were grown for about eight months in a medium containing $2x10^{-6}$ M Dex. Cells of clones D-2 and D-F were then divided and grown either in the présence or in the absence of Dex. Cells from clones D-2 and D-F grown without Dex were designated clone 2 and clone F .

Subclones of these variants (2-28, 2-29, Fα5, Fα6) were derived by isolating colonies after single-cell plating.

This variants had reduced Dex-sensitivity. The differentiated Fα6 subclone expressed most of the liver-specific functions found in the Faza 967 cell line and the secretion of AFP (Alpha-fetoprotein) activated in this cell line (A. Venetianer Differentiation 32:148-156 1986).

2.1.2 Media and culture conditions

The cell line were cultived in F-12 media (provided in powder form by Grand Island Biological Co.), supplemented with 5% fetal calf serum (Gibco).

The cells were grown in plastic tissue-culture bottles or in plastic or glass Petri dishes in a humidified atmosphere of 8% CO_2 and air at 37^0C.

The cells were detached for subculture and collected for enzyme assay with a phosphate-buffered saline solution containing 0,02% EDTA and 0,05% trypsin.

2.1.3 Cell fusion and selection of hybrid clones

The parental cell lines were grown for approximately 21 days in F-12 medium supplemented with FCS 5%, 10^{-4}M BUdR for the TK- line and 10^{-4} M 2-Amino-6-Mercantopurine for the HPRT- line, to eliminate possible revertants.

After 21 days, $1x10^{6}$ cells from each parental line are centrifugated together and maintained in cell culture during 24 hours at 37°C in 30 mm Ø Petri dishes in F-12-FCS medium.

The fusion agent was Polyethyleno Glycol 1000 (PEG) (for cromatografia in gas, Merk) in concentration of 50% PEG wt/wt in serum-free F-12 medium.

After 24 hours of cocultivation the confluent cell monolayer was washed 3 times with F-12 and treated with 2 ml of 50% PEG for about 45-60 seconds, the PEG was then aspirated and the cells were rinsed six times with F-12 serum-free medium.

The culture was incubated for about 270 minutes in F-12-FCS, to permit the recovery of the PEG-treated cells and to allow the convalescence of cells that had fused. After that, the cells were detached in the following way : 1 ml of 0,25% trypsin, EDTA was added for 1 minute, diluted with 1 ml of 0,05% trypsin, EDTA and the cells removed with F-12-FCS-HAT (HAT: Hypoxanthine Aminopterin Thymidine). The HAT selective medium is : Hypoxanthine 10^{-2} M, Aminopterin $4x10^{-5}$ M, Thymidine $1,6x10^{-3}$ M (Littlefield J. Science vol.145: 709-710 1964).

The cell suspension was divided into five Petri dishes 140 mm Ø, $3-5x10^{5}$ cells per Petri dishe and maintained in culture in F-12-FCS-HAT selective medium until the cloning of the cells.

Simultaneously with the cocultivation of the parental cell lines, we started a culture from each with $1x10^6$cells in 25 ml plastic bottles of for testing possible revertants. The F-12-FCS medium was changed to the selective medium, when the selection for hybrid cells was initiated (A. Venetianer et al. Acta Biol. Acad. Sci. Hung. 32, n 3-4:175-187 1981).

After 14 days, when the cell clones were well identifiable the cells are released by trypsin treatment with the aid of sterile stainless-steel cylinder, and kept in culture in 30 mm Ø Petri dishes in F-12-FCS-HAT (Isolation of clone strains Ann.Rev.Enzy. vol.9: 107-108). When enough cells were available they were transferred to 25 ml plastic tissue-culture bottles kept in culture definitively in F-12-FCS-HAT selecting medium.

2.1.4 Chromosome preparations

Cultured cells in log phase were maintained for 16 hours in F-12-FCS medium with 10^{-7}M Methotrexate, washed three times with PBS and maintained for another six hours in F-12-FCS medium with 10^{-5}M Thymidine. Posteriorly the cells were treated with Colcemid 0,04 µg/ml during 15 minutes. The cells were harvested by trypsinization and the chromosome preparations were made by the usual air-drying procedure (Schnedl W. Nature New Biol.233: 93-94 1971).

The chromosome preparations obtained were stained with 5% Giemsa posterior to a short treatment of 45"-60"with 0,5% Trypsin, in PBS, to obtain the characteristic banding patterns of the rat chromosomes.

These preparations were used for the control of cell fusion chromosome analysis and determination of mean chromosome number of different cell lines (Jorge J. Yunis et al. Chromosoma (Berl) vol.67: 293-307 1978).

2.1.5 Azacytidine treatment

Different numbers of cells ($5x10^6$ for AFP and Alb, $1,5x10^6$ for TAT determinations) were incubated in F-12-FCS-HAT. 24 hours later, exponentially ... growing culture were treated with 5-Azacytidine in PBS, sterilized by filtration and dissolved freshly for each experiment (Hickey I. et al. Exp.Cell Res. 164: 251-255 1986). Different concentrations of 5-Azacytidine were used in the treatments, beginning with 0,5 µg/ml to 14 µg/ml. After different treatment times, (between 20-48 hours) the cell monolayers were washed 3 times with sterile PBS. The cells were re-fed with medium without 5-Aza and maintained in culture during different periods (20-48) hours. After these periods the cell samples were used for the determination of AFP, Alb and TAT and for DNA preparation.

Similar cell cultures were used in order to determine the survival of these cells in the absence and in the presence of the same 5-Aza. concentration used in the treatments. This was determined because 5-Aza. may have an effect over cellular metabolism in several different ways and there is no unquestionable evidence that the inhibition of DNA methyltransferases is, in fact the only or the major influence on gene activity of this cytosine analogous (Jones A. P. et al. Proc. Natl. Acad. USA

vol. 79: 1215-1219 1982). A toxic action at high concentrations is also present.

2.1.6 Plating test or Colony formation

This technique was used for the determination of steroid Dex. sensitivity of the cell lines.

Triplicated cell cultures were made with a given number of cells (700 cells in o,2 ml) for each concentration in plastic Petri dishes of 60 mm ∅ in F-12-FCS-HAT medium for the control and medium containing 10^{-6} M Dex. and 10^{-7} M Dex. After a week, the culture medium was changed and after 14 days of culture the cells were fixed with 5% of Formaline in PBS, having been previously washed with PBS and after wards stained with 1% of Gentian Violet or with 5% Giemsa.

The plating efficiency and the colony size were analized by comparing the number and the size of colonies in different Petri dishes (control and treated) and the results are given in percentage, taking control as 100% (A.Venetianer et al. Som.Cell Genet. vol.4,N5:513-530 1978).

To characterize a given clone three broad classes were designated :

- resistant, no effect of Dex could be detected by one /r/ or both /rr/ tests
- sensitive /s/, both plating efficiency and colony size were strongly reduced by Dex, >41% of the control
- intermediate /i/, redution between 5-40% of the control

It could appear some cases of resistant cell lines having

high plating efficiency, in Dex presence, but the colonies being smaller than in the control dishes. In such cases the two parameters will give different levels of sensitivity (ri or si).

2.2 Biochemical techniques

2.2.1 Steroid binding by whole cells

High density cell cultures were harvested by tripsinization and washed three times with cold PBS.

The final cell pellet was resuspended in such a way as to leave $2x10^6$ cells in 100 µl of F-12.

For measuring steroid uptake, series of 100µl cell suspension in F-12, were incubated together with 100 µl of F-12 containing [3H] Dex. in different concentrations, between $10x10^{-8}$ M and $0,625x10^{-8}$ M and in the absence or presence of $1x10^{-5}$ M competing unlabelled Dex.

After 40 minutes of incubation at 37^0C, the cell suspension was washed 3 times with cold PBS, in order to remove the excess of unspecifically bound [3H] Dex.

The cell pellets were dissolved directly in scintillation liquid (Tritosol PPO 3,0 g, Triton X-100 257 ml, Ethylenglycol 37 ml, Ethanol 106 ml in 1000 ml Xylol) for the determination of receptor-associated radioactivity, using a Packard Tricarb liquid scintillation counter.

The amount of specific binding was taken as the difference between parallel samples, one incubated with labelled hormone only and the other with both, labelled and unlabelled hormone

(A.Venetianer Som.Cell Genet. vol.4,N5:513-530 1978).

2.2.2 Indirect immunofluorescent staining of AFP and Alb

This method is used for staining the AFP and Alb producing cells.

The immunofluorescent staining is based on the fixed cells treatment with antisera against rat AFP and Alb on glass coverslips, after rinsed to remove the unspecifically bound antibodies, and treated with sera containing species-specific antiglobulin conjugated with fluorescein.

The staining of the Alb and AFP synthetising cells, resulted in a bright staining of the Golgi apparatus, where Alb and AFP are concentrated before secrection.

The staining was performed according to the method of Mevel--Nino and Weiss (Mevel-Nino N. and Weiss M.C. Jour.Cell Biol. vol.30 : 339-350 1981), 24 hours cell cultures, $5x10^4$ cells on glass coverslips were used for the staining in each series of experiments.

Formaldehyde, methanol fixation method was used.

50 µl/coverslip of the antisera dilution against rat AFP obtained in sheep (Nordic Immunologi, Plasma protein antisera Sh ARa/AFP) in PBS, was used for AFP detection and antisera against rat Alb obtained in rabbit (Nordic Immunologi, Plasma protein antisera R ARa/Alb) for Alb detection.

The bound immunoglobulines were detected with rabbit fluorescein-conjugated globulins directed against sheep IgG (Nordic Immunologi S Ash/FITC) in PBS, 50 µl/coverslip for AFP and

sheep fluorescein-conjugated globulins directed against rabbit IgG (Inst.Pasteur Production, Globulines Fluorescentes du Mouton anti Ig de lapin) in PBS 50 µl/coverslip for Alb.

We counted at least 250-300 cells per coverslip, a percentage of positive cells per total counted cells was calculated.

2.2.3 Determination of Tyrosine aminotransferase activity

The technique for determination of the TAT activity was based on the transformation of p-hydroxyphenylpyruvic acid into p-hydroxybenzaldehyde.

Two parallel cell cultures initiated with $2x10^6$ cells in Petri plastic dishes of 100 mm Ø, were incubated and 48 hours later, they were kept for 16-18 hours in fresh medium with $1x10^6$ M Dex. (induced TAT) and without Dex. (basal TAT). The cell extracts from this cell cultures were prepared as described by Schneider J.H. et al.(Proc. Natl. Acad. Sci. USA vol.68: 127-131 1971).

The TAT activity was assayed using the method of Diamonstone T.I.(Analy. Bioch. 16: 395-401 1966) as was described by Hayasmi S. et al.(Jour. Biol. Chem.242 :3998 1967).

Briefly, dilutions of the cell extracts were used for the determination of TAT activity. For each sample three reaction tubes were done A,B,C. Tube A is the blank, B and C were two parallel reaction tubes.The reaction was carried out at 37°C for 15 minutes and stopped by adding 100 µl of 10 N KOH. 30 minutes later the solutions were read in the SP6-500UV spectrophotometer (PYE UNICAM) at 331 nm against control .

The Specific Enzimatic Activity ESA mU/mg protein, was calculated by using the empirical formula :

$$\frac{O.D \times 10}{15 \text{ min.} \times 20}$$

One milliunit of TAT activity catalyses the formation of 1 µmole of p-hydroxyphenilpyruvate per min. at 37°C.

The TAT activity was determinated at least twice from the cell extract.

2.2.4 Determination of protein concentration

The method of Lawry O.H. et al. (Jour. Biol. Chem. 193: 265-275 1951) was used for the determination of protein concentration in the cell extracts used for the determination of TAT activity.

Briefly, for the standard curve bovin serum albumin was used 5,10,15,20,40 µg. The reaction was performed on the same cell extracts used for the determination of TAT activity. Three reaction tubes A,B,C; A blank, B and C parallel tubes with different dilutions. The reaction was carried out at room temperature for 15 min. and stopped by adding 100 µl of Folin 1N. 30 minutes later the reactions were read in the same spectrophotometer as in the case of TAT at 750 nm, against control.

2.2.5 Electrophoretic analysis of ADH, LDH, Ald

These isozymes were detected through electrophoresis.The cell samples were obtained from cell cultures with $2x10^6$ cells in plastic Petri dishes. The cells were harvested by tripsinization and washed several times with PBS (Bertolotti R., Weiss M.C. Biochem. 54: 195-201 1972).

The cell extracts were prepared by breaking the cells in extraction buffer by 3 cycles of freezing-thawing. The cell debris was separated from the cell extract by centrifugation in Beckman air fuge at 30 psi during 5', the clear supernatant served as cell extract.

These isozymes were detected by electrophoresis on cellulose acetate strips (Cellogel: gelatinized cellulose acetate strips Chemetron Milan) and staining the cellogel strips with the staining mixtures prepared in 5% agarose.

The different electrophoresis conditions and staining mixtures were :for the ADH as was described by Bertolotti R. et Weiss M.C. (Bioch. 54:195-201 1972); for the Ald as described by Bertolotti R. et Weiss M.C.(Jour.Cell Physiol. 79:211-224 1972) and Penhoet E. et al.(Proc. Natl. Acad. Sci. USA 56: 1275-1282) and for the LDH as described by Meera K. et al. (Arch. Biochem.Biophys. 145 : 470-483 1971).

2.3 Molecular techniques

The molecular techniques include DNA and RNA preparations, Southern and Northern blots, determination of DNA methylation by restriction enzymes and complementary techniques.

2.3.1 Preparation of DNA

DNA from different cell lines was prepared following two different methods.

Method I : DNA was prepared from isolated nuclei. The nuclei isolation was carried out as described by Tretner J. et al.(Mol. Cell Biol. vol.7: 1856-1864 1987).

The pelleted nuclear sediment was resuspended in digestion buffer as described by Tretner J. and incubated over night at 37°C with proteinase K 250 µg/ml. The following steps were made approximately as was described by Sala-Trepat J.M. et al. (Proc. Natl. Acad. Sci. USA vol.76: 695-699 1979).

After the over night digestion, a first phenol extraction of the solution was made with 2 times the volume of phenol, 2 times the volume of chloroform/isoamyl alcohol and finally with 2 volume of ether. The ether was removed from the aqueous fase and digested with RNase 200 µl/ml at 37°C 3 hours. After this time SDS was added in a concentration of 0,5% and proteinase K 50 µg/ml and left to digest over night.

After a second extraction with phenol, the DNA was precipited with ethanol -20°C and left at -20°C. After successive ethanol washing, the dry DNA pellet was resuspended in TE pH7,5 TRIS-HCl

10 mM pH7,5, 1 mM EDTA.

Method II : DNA from the CsCl gradient (see isolation of total cellular RNA) was recovered and prepared. The DNA after ultracentrifugation was found in the lower 1/3 part of the CsCl gradient and was removed and precipited with ethanol at -20°C. After successive rinsing with 70% ethanol to eliminate the CsCl, the dry pellet was resuspended in TE buffer and left there several days to dissolve.

The phenol extraction of this solution and the following steps were already described.

2.3.2 DNA methylation analysis and Southern blots

The isoschizomer restriction endonucleases Hpa II and Msp I for the examination of the methylation stage of internal cytosine in the CCGG sequences of the Alb and AFP genes were used.

Whenever the internal C residue is methylated the Msp I will cleave it, however Hpa II cannot cleave.

80 µg from the isolated DNA samples were digested with different restriction enzymes in order to delimit the examined DNA fragment. Usually with Eco RI, and/or Hind III single or double digestions were done. These digested samples were divided and one of them, corresponding to 40 µg DNA, was digested with Hpa II and the others 40 µg DNA with Msp I. All digestions were carried out at 3 units of enzyme/µg DNA at 37°C overnight and after each digestion minigel control was performed.

The restriction fragments were separated on 1% agarose gel in

TEB buffer. A DNA size standard was included also.

The DNA in the gel was depurinated with 0,25 N HCl 15 min., denaturated with 1,5 M NaCl 0,5 N NaOH 2 hours and neutralized with 0,5 M TRIS pH 7,0, 2 M NaCl. After DNA transfer to nitrocellulose (Schleicher & Schuell) as described by Southern E.M. (Jour.Mol.Biol. 98: 503-517 1975), the blots were baked in a vacuum oven for 2 hours at 80^{0}C and stored until hybridization.

The nitrocellulose blots were washed 30 min. at 66^{0}C in 5xSSC and 30 min. at 66^{0}C in 5xSSC, 10xDenh. before the prehybridization.

Nitrocellulose blots were prehybridized overnight at 66^{0}C in plastic bags in prehybridization solution containing 5xSSC, 10xDenh., 50 mM TRIS-HCl pH 7,5, 1 mM EDTA, 0,1% SDS, 500 µg/ml sonicated and denaturated chicken DNA.

The blots were hybridized with random oligolabelled [32P] DNA probes (see random oligo-labelling) in the same prehybridization buffer, 24 hours at 66^{0}C.

Following the hybridization, the nitrocellulose blots were washed once in 2xSSC, 1xDenh, 0,1 SDS, 0,1% NP 30 minutes at 63^0 C, once in 1xSSC, 1xDenh, 0,1% SDS, 0,1% NP 30 minutes at 63^{0}C and in 0,7xSSC, 0,1% SDS, 0,1 NP 30 min. at 63^{0}C.

The blots were then wrapped in plastic bags and exposed to X-ray film for 10-15 days at -70^{0}C, using intensifying screens.

2.3.3 Isolation of total cellular RNA

Total cellular RNA from different cell lines was prepared by the method of Chirgwin J.M. et al.(Biochemistry vol.18:5294-5299

1979) and described by Maniatis T. and Fritsch E.F.(Molecular cloning,lab manual pp194-197 Cold Spring Harbor Lab.pub.1982) as Guanidine thyocyanate/CsCl method.

Succinctly : cell monolayer cultures were lysed by addition of the guanidine thyocyante solution, approximately 8 ml of cell lysate was layered into ultracentrifuge tubes previously filled with 3 ml of 5,7 M Cesium Chloride and centrifugated in Beckman L2-65B ultracentrifuge, rotor SW 41 at 16^{0}C,16 hours, 33000 rpm.

This method is based on the fact that the bouity density of the RNA in CsCl solution is higher then any other macromolecule. After the ultracentrifugation, the RNA forms a pellet at the bottom of the tube, the DNA and proteins move up on the CsCl solution.

The RNA pellet in the bottom of the tube was dissolved in dissolving buffer and after the quantity and quality control of the prepared RNA, was precipited with ethanol at -20^{0}C and kept at -70^{0}C.

2.3.4 Northern-blot analysis

The Northorn-blots were carried out according to Gal A. and co-workers (Analy.Biochem. 132:190-194 1983) with slight modifications.

Briefly : the precipited RNA samples were washed with ethanol and the dry RNA pellet redissolved in sample buffer containing : 50% desionized Formamid and 2,2M formaldehyde in sodium phosphate buffer. These RNA samples were dialized against the sample buffer and 25 µg RNA of these samples were maintained at 65^{0}C

for 5',before the electrophoresis in agarose gel 1,2% agarose, 2,2 M formaldehyde in phosphate buffer according Rave N. et al. (Nuc.Acid.Res. 6:3559-3567 1979).

The transfer of the RNA's from the agarose gel to the nitrocellulose filter(Schlecher and Schull) was performed essentially as described by Thomas P.S.(Proc.Natl.Acad.Sci.USA 77:5201-5205 1980).

The filter-bund RNA was prehybridized 12-24 hours at 42°C and the prehybridization solution contains : 50% Formamid, sodium phosphate buffer 50 mM pH 6,5, NaCl 0,7 M, EDTA 4 mM, SDS 0,08%, Denhardt 8x, sonicated denaturated chicken DNA 0,5 mg/ml. The hybridization was made in the prehybridization solution at 42°C, 24 hours, containing the [32P] labeled DNA probe. The DNA probe, the plasmid DNA preparation and the [32P] labeling of the DNA probes will be described later.

In each case, the used labelled DNA probes will be mentioned under the presented figures.

After the hybridization, the nitrocellulose filters were washed in the following way :

- twice at 42°C with 50% formamid, NaCl 0,9 M,sodium phosphate buffer 50 mM pH 7,0, EDTA 5mM, SDS 0,1%, Denhardt 1x
- twice at 46°C with 50% formamid, sodium phosphate buffer 50 mM pH 7,0, EDTA 5 mM, SDS 0,1%
- once at 52°C and once at 63°C with 0,1% SDS and 0,1xSSC

The nitrocellulose filters were exposed to X-ray film with intensifying screen at -70°C.

2.3.5 Molecular hybridization probes

Two types of molecular probes were used: first, cDNA probes, complementary to rat AFP mRNA pRAFP65, pRAFP87, Fig.2 A (Jagadzinsky L.C. et al. Proc.Natl.Acad.Sci. USA 78:3511-3525 1981) to rat Alb mRNA pRSA13, pRSA57, Fig.1 A (Sargent T.D. et al. Proc. Natl. Acad.Sci.USA 78:1180-1183 1981), to rat TAT mRNA pcTAT-3, pcTAT-2, Fig.3 (Shinomiya T. et al. Proc.Natl.Acad.Sci.USA 81: 1346-1350 1984), to rat receptor mRNA pSG1, a 2,2 kb XbaI-PstI fragment inserted into pSP65, this clone is a derivative of pRM16 which was described by Miesfeld R.L. et al. (Nature 312: 779-781 1984) and to rat Glyceraldehyde 3-phosphate dehydrogenase mRNA (Tso Yun J. et al. Nuc.Acids Rese. 13:2485-2502 1985), and secundly, genomic DNA probes, corresponding to genomic subclones of the rat Alb gene Sub JA, Sub JB Fig.1 A (Sargent T.D. et al. Proc. Natl. Acad. Sci.USA 78 :1180-1184 1981) to the rat AFP gene, genomic probes PO1, PO2, PO3, PO4, Fig. 2 A obtained from the PO EcoRI fragment by subcloning (Gal A. et al. Differentiation 29:238-242 1985). These probes are specific to 5' flanking sequences.

All these probes were inserted in plasmids.

2.3.6 Isolation of plasmid DNA

The preparation of plasmid DNA containing the inserted probe DNA, was made from Chloranfenicol 170 µg/ml amplified bacterial cultures. The preparation of bacterial lysat is similar to the described by Clewell D.B. and Helinski D.R. (Proc.Natl.Acad.Sci.

USA 62:1159-1166 1969).

Briefly, the bacterial cell pellet was resuspended in TE buffer and freshly dissolved Lisozyme was added to the bacterial suspension and maintained for 5 min. at 0°C.

After adding EDTA pH 8,0 in a concentration of 100 mM, was kept for another 5 min at 4°C, after which lysis was brought about by adding Triton X100 detergent mixture consisting of the following:- Triton X100 0,1%, EDTA pH 8,0 62 mM, and TRIS pH 8,0 50 mM, after 20 min. at 0°C, the sample becames relatively clear. The lysat was centrifugated in rotor JH-20 at 18000 rpm 50 min.

The RNA from the sample was digested by adding to the supernatante RNase 20 µg/ml and left at 37°C, 2 hours.

After the extraction with phenol/cloroform, the plasmid DNA was precipitated by adding 0,1 volume of 2 M NaCl and 2 volumes of Ethanol -20°C. After a second precipitation, similar to the previous one, and successive rinsing with 70% ethanol and absolute ethanol, the final plasmid DNA solution can be further purified by two different techniques.

Spermin precipitation. To the plasmid DNA solution Spermin was added in concentration of 10 mM and maintained for 30 min at 0°C. After the centrifugation at 4000 rpm 20 min at 0°C, the plasmid DNA was precipitated again, and finally the dry plasmid DNA pellet was resuspended in TE buffer and controlled in minigel.

This spermin purified plasmid DNA can be used as it is, or purified in a second step by centrifugation to equilibrium in Cesium chlorid-Ethidium bromide gradients as was described by

Maniatis T. and Fritsch E.F. (Molecular cloning, lab.man. 93-95, Cold Spring Harbour Laboratory, publ. 1982).

2.3.7 Random oligo-labelling techniques

The purified plasmids were linearized with different restriction enzymes and were labelled with [32P]-deoxyribonucleotides by the random oligo-labelling technique, using the Klenow fragment of DNA polimerase I as was described by Feinberg A.P. and Vogelstein B. (Analy.Biochem. 132:6-13 1983).

3. RESULTS AND DISCUSSION

3.1 Expression of AFP, Alb, LDH, Ald and TAT in hepatoma cell hybrids

Cell hybrids were made between two cell lines phenotypically different H56 (AFP-, Alb-, Ald-B-, ADH-, LDH-β- and Fα6 (AFP+, Alb+, Ald-B+, ADH-, LDH-, B+).

38 hybrid cell clones were isolated from 5 different plastic Petri dishes. Chromosome analysis of the isolated hybrid clones revealed that they contained chromosomes from both parental cell lines.

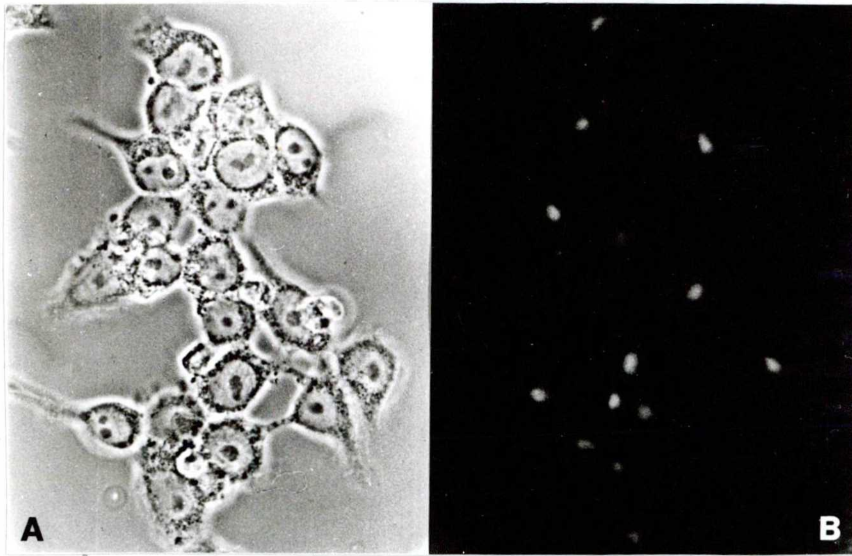
The mean chromosome number of the hybrid clones was about 94 and the parental cell lines H56 43(mean), 40-45(range) and Fα6 6,51(mean).

All 38 isolated clones were tested for AFP and Alb production by the indirect immunofluorescent staining and all clones were found to be negative referring to AFP and Alb production. Fig. 8 shows indirect immunofluorescent staining of Alb and AFP in parental cell line.

Five different cell clones were checked periodically (each month) for the AFP and Alb prodution during seven months. During this period no re-expression of the two seroproteins was found.

The same five cell hybrid clones were checked for Ald, ADH, LDH production by electrophoresis on cellulose acetate strips. Fig. 9 shows zymograms from the parental cell lines and cell hybrid clones. In the Fα6 parental cell line Ald A and B production could be detected in the form of A4 homotetramer and

I



II

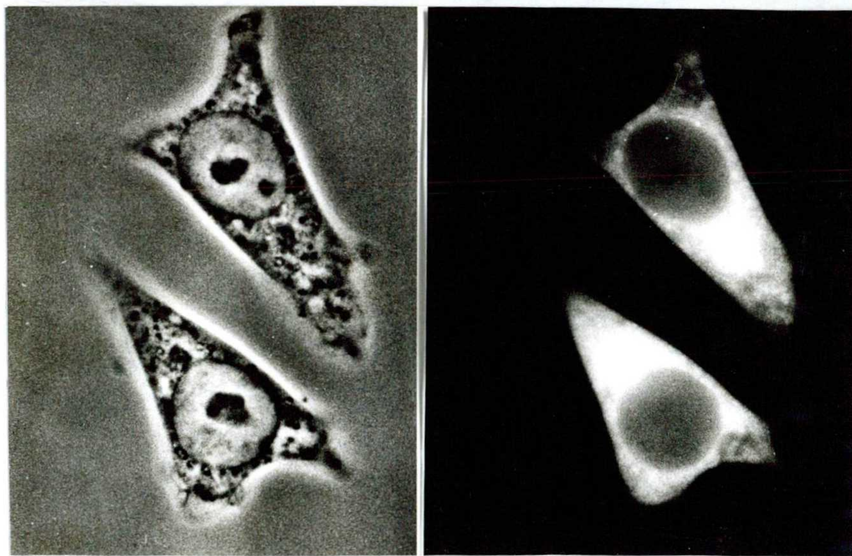


Fig. 8 . I and II indirect immunofluorescent staining Alb and AFP.

I Immunofluorescent preparation stained by rabbit antibodies to rat serum Alb.

II Immunofluorescent preparation stained by sheep antibodies to rat AFP.

A. Phase-contrast optics; B. Epifluorescent optics

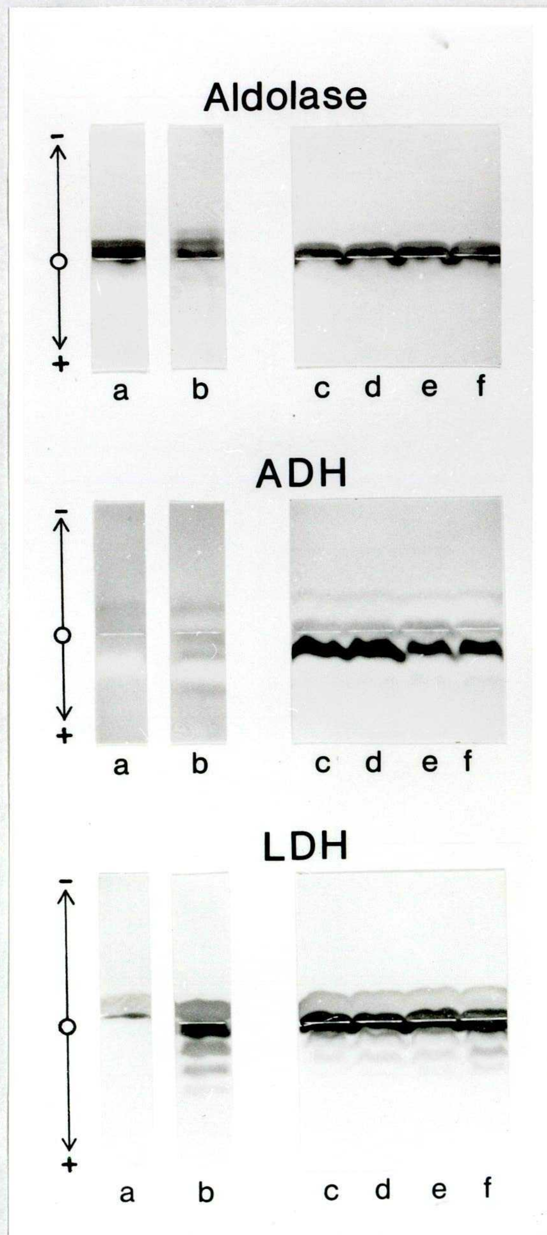


Fig. 9 . Isozyme pattern of Ald, ADH, LDH from parental and hybrid cell lines. a) H56; b) Fα6; c) VIII-2-12; d) VIII-3-20; e) VIII-3-23; f) VIII-4-29.

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A-B heterotetramers. Traces of ADH-L production could also be detected and five isozymes of LDH could be visualized upon electrophoresis. In the second parental cell line H56, Ald-A production could be detected and only LDH-A could be visualized upon electrophoresis.

The four cell hybrid clones presented show an almost similar pattern of isozymes. The presence of Ald-A, absence of Ald-B, presence of ADH-S and five forms of LDH could be visualized upon electrophoresis.

Tab. 1 shows the observed basal and induced TAT levels in the parental and hybrid cell lines. All the presented cell lines were TAT negative. A TAT positive Faza 967 cell line, is also presented.

In order to obtain re-expression of AFP and Alb genes, several cell clones, (two cell hybrid clones VIII-2-12, VIII-2-23, Clone 2 cell line and another cell line named Fα5, which had lost the AFP production, but not the Alb production, two months before the treatment) were treated with 5-Azacytidine. The treatment conditions and the results are presented in Tab.2. The treated cell lines were checked for AFP and Alb production and no re-expression of the AFP and Alb genes were found. In order to research another function, the Clone 2 cell line was treated with 5-Azacytidine and TAT activity was determined. Similar to AFP and Alb no re-expression of TAT gene in Clone 2 cell line was found(data not shown).

The impossibility to obtain re-expression of liver-specific functions such as AFP, Alb and TAT, by-Aza. treatment is in agreement with results obtained in other laboratories.

Tab. 1. Basal (B) and induced (I) TAT activity from parental and hybrid cell lines. I/B is the rate between the induced and basal values.

Cell line	T A T (b)			Time after fusion(days)
	B	I	I/B	
Parents				
Fα6	3,5	21,6	6,1	44
H56	3.08	3,72	1,2(a)	95
Hybrids				
VIII-2-12	4,02	9,86	2,45	81
VIII-3-20	5,51	7,81	1,42(a)	63
VIII-3-22	7,959	13,64	1,71	65
VIII-2-23	4,39	7,62	1,74	81
VIII-4-29	5,85	8,14	1,39	81
Others				
Faza-967	61,6	366,45	5,95	-

(a) average of two samples; usually for each sample the given value in the table is the average of 3 measurements.

(b) the presented value is "Specific enzyme activity" express in mU/mg protein

Tab.2. Treatment conditions of the cell lines with 5-Aza.
See Aza. treatment (Materials and methods)

Cell line	5-Aza. µM/ml	Time of treat. (h)	Time after treat. (h)	I.F. staining of AFP. Alb. before treat.		after treat.	
				AFP	Alb	AFP	Alb
Hybrids							
VIII-2-12	0,5	30	30	N	N	N	N
	1	30	30	N	N	N	N
			60	N	N	N	N
VIII-3-23	0,5	30	30	N	N	N	N
	1	30	30	N	N	N	N
			60	N	N	N	N
	2	25,5	20,5	N	N	N	N
	3	25,5	20,5	N	N	N	N
	4	25,5	20,5	N	N	N	N
	5	25,5	20,5	N	N	N	N
	6	25,5	20,5	N	N	N	N
	7	60	>20	N	N	N	N
		30	<20	N	N	N	N
			>20	N	N	N	N
	8	30	<20	N	N	N	N
			>20	N	N	N	N
	10	30	<20	N	N	N	N
			>20	N	N	N	N
	12	30	<20	N	N	N	N
			>20	N	N	N	N
	14	30	<20	N	N	N	N
			>20	N	N	N	N
Faza 967	0,5	24	24	N	P	N	P
	1	24	24	N	P	N	P
			48	N	P	N	P
Clone 2	0,5	24	24	N	N	N	N
	1	24	24	N	N	N	N
			48	N	N	N	N
	2	24	24	N	N	N	N
	3	24	24	N	N	N	N
	4	24	24	N	N	N	N
	5	24	24	N	N	N	N
	6	24	24	N	N	N	N
	7	24	>20	N	N	N	N
			<20	N	N	N	N
	8	48	>20	N	N	N	N
		24	>20	N	N	N	N
			<20	N	N	N	N
	10	24	>20	N	N	N	N
			<20	N	N	N	N
	12	24	>20	N	N	N	N
			<20	N	N	N	N
	14	24	>20	N	N	N	N
			<20	N	N	N	N
Fα5 (a)	3	24	16	N	P	N	P
	6	24	16	N	P	N	P
	7	24	16	N	P	N	P
	9	24	16	N	P	N	P
	12	24	16	N	P	N	P
	14	24	16	N	P	N	P

(a)This line lost AFP prodution two months before treatment
N - non-production ; P - production

In conclusion we have shown :

- extinction of AFP and Alb genes and inability to be re-expressed by 5-Azacytidine treatment in our study conditions
- extinction of Ald-B and maintenance of the non hepato-specific forms Ald-A, and the five forms of LDH and re-expression of ADH-S.
- absence of TAT activity in the hybrid cell lines.
- a total stable and co-ordinated extinction of hepato-specific functions but not of non hepato-specific functions expressed in these hepatoma cell lines.

To our knowledge, it was the first time that such a complete, total and stable extinction of AFP and Alb was obtained in hybrids between hepatomas.

3.2 Expression of AFP, Alb and TAT at RNA level

The hepato-specific functions are mainly regulated at transcriptional level, as was shown by other studies (Venetianer A. et al. Differ.32:148-156 1986; Nahon J-L. et al. Nuc.Acid.Res. 10:1895-1911 1982).

These data together with data obtained by the biochemical analysis of the cell hybrids suggested, but did not prove, that some of this examined hepato-specific functions are regulated at the transcriptional level. One question that should be put is at what level is the regulation of the expression of AFP and Alb genes in hybrid cell lines made?

We performed the analysis of the specific mRNA sequences.

Northern blot analysis of AFP mRNA sequences in total RNA

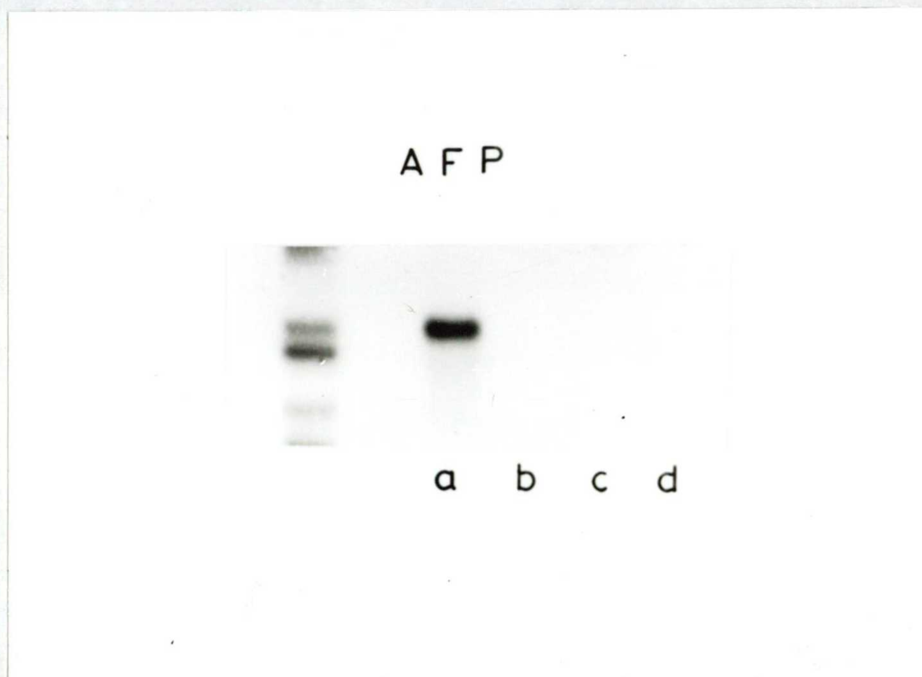

Fig. 10 . Northern blot analysis of AFP mRNA. 25 μg of total RNA per lane, were hybridized with AFP cDNA probes pRAFP65, pRAFP87. Lane a. Fα6; b. H56; c. VIII-3-22; d. VIII-3-23.

preparations, are shown in Fig.10. It can be seen that only the Fα6 AFP + parental cell line gave a signal corresponding approximately to 2,3 kb AFP mRNA. The other parental H56 as well as the hybrid cell lines VIII-3-22, VIII-3-23 do not give any signal corresponding to the AFP mRNA even after a long exposition. In order to check the RNA quantity and quality, to prevent false results, the same nitrocellulose filter was hybridized with rat G3PDH specific cDNA prebes. No differences in the G3PDH mRNA (used as control) were observed among the parental and hybrid cell lines (data not shown).

Fig.11 shows Northern blot analysis of Alb mRNA sequences in total RNA preparations, hybridized with two Alb cDNA probes. Similar to the AFP, only the Alb+ parental Fα6 cell line gave signal corresponding to the approximately 2,3 kb Alb mRNA. No signal corresponding to the Alb mRNA was detected in the other cell lines even after a long exposition. Again no differences.

Northern blot analysis of the TAT- parental and hybrid cell lines showed that no TAT mRNA could be detected even after a long exposition in these TAT- cell lines.

As a conclusion, it can be said that the regulation of AFP and Alb is at transcriptional level in the hybrid cell lines.

The extinction of AFP and Alb in the cell hybrids is at transcriptional level, most probably from a transcriptional block, similar to the parental cell lines.

3.3 Methylation analysis of the 5'region of AFP and Alb genes

Previous Southern blot analysis of digested DNA from diffe-

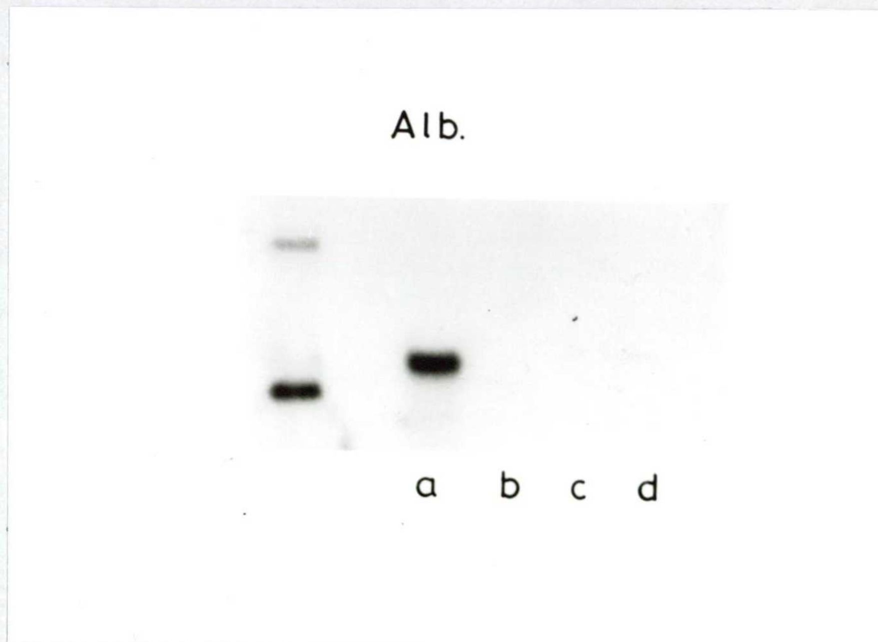

Fig. 11 . Northern blot analysis of Alb mRNA. 25 μg of total RNA per lane, were hybridized with Alb cDNA probes pRSA13, pRSA57. Lane a. Fα6; b. H56; c. VIII-3-22; d. VIII-3-23.

rent parental and hybrid cell lines indicated that the gross organization of the AFP and Alb genes were unchanged in these cell lines (data not shown). Based in these results it could be excluded that gross rearrangement, insertion or deletion is responsible for extinction of these genes in cell hybrids.

The level of methylation of the internal Citozine in the sequences CCGG at 5'region of AFP and Alb genes, in the parental and hybrid cell lines were determined by digestion with isoschizomer restriction endonucleases HpaII and MspI.

In several studies correlation were found between the hypomethylation of some specific sites and the expression of genes. Two such MspI sites M 0, M-2 (hypomethylated on the internal Citozine) in the 5'region of AFP gene Fig.2 B, and one M+1 in the 5'region of Alb gene Fig.1 B, were found in the AFP and Alb producer cells (Tratner J.et al.Mol.Cell.Biol.7:1856-1864 1987).

The principal question that should be put is whether the extinction of AFP and Alb gene expression in the cell hybrids is correlated or not with alterations of the methylation pattern in the 5' region of AFP and Alb genes?

3.3.1 Methylation pattern of the 5'region of AFP gene

Fig.12 shows the methylation state of the 5'region of AFP gene at M 0 site mapped into the first exon, about 30 nucleotids downstream from the TAT box. The autoradiogram was obtained with probe P04 and this revealed the level of methylation of the site M 0 (see Fig.2 B). The AFP+ parental cell line shows two bands with similar M.W. of about 1,0 kb. This corresponds to the

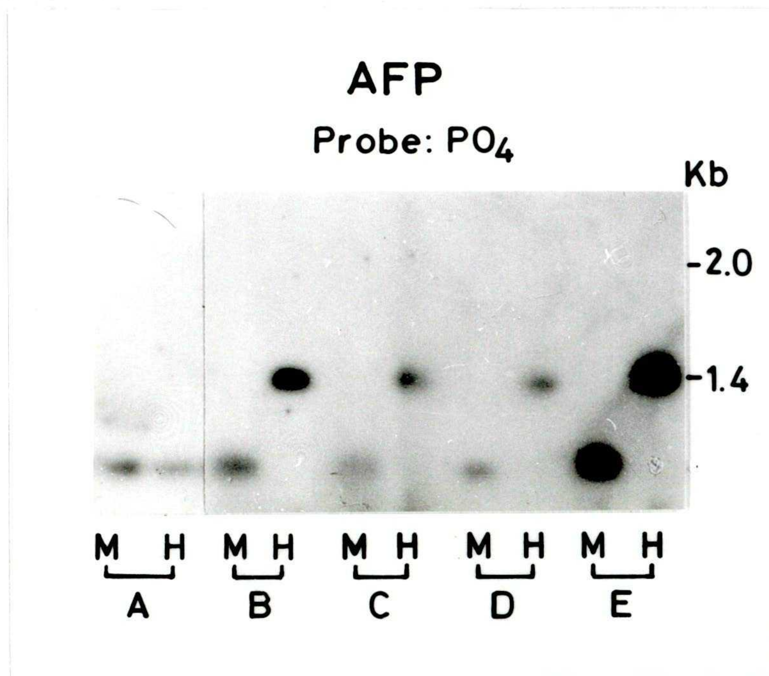

Fig. 12 . Methylation state of the 5'region of the rat AFP gene, site M 0. Genomic DNA digested with Eco RI -Hind III and then either by Msp I (M) or Hpa II (H) as described in Materials and Methods. Hybridized with probe PO-4. Lane A. Fα6; B. VIII-3-23; C. VIII-2-12; D. VIII-4-29; E. H56.

demethylated state of the site M 0 (HpaII cut only when the internal Citozine in the sequence CCGG is demethylated). Lane E. the AFP- H56 parental cell line, two bands with different M.W. can be seen. The higher M.W. band 1,4 kb corresponds to the fragment obtained by the double EcoRI-HindIII digestion, because the M 0 site can not be cut by HpaII when the internal Citosine is methylated. The lanes B.C.D. correspond to three hybrid cell lines and the observed methylation pattern is similar to the AFP- H56 parental cell line.

The Fig.13 shows the methylation state of the 5'region of AFP gene at site M-2 located upstream of the M 0 site (see Fig.2 B). A similar pattern of methylation as in the case of M 0 site was obtained.

Two bands of the same size were obtained in the case of AFP+ Fα6 and two bands with different size were obtained in the AFP- H56 parental and in three hybrid cell lines.

The higher band corresponds to the EcoRI-HindIII fragment because the HpaII restriction enzyme will not cut when the internal Citozine in the M-2 site is methylated.

The methylation state of this two M 0 and M-2 sites seems to be well associated with AFP gene expression in the cell hybrids, similar to the parental cell lines as well as in other cell lines and tissues. These sites were undermethylated in the AFP producer cell lines and fully methylated in the AFP nonproducer parental and hybrid cell lines.

In contrast to these sites the methylation state of other sites (see Fig.2 B) located in the 5'region of AFP gene were not associated with AFP gene expression (data not shown).

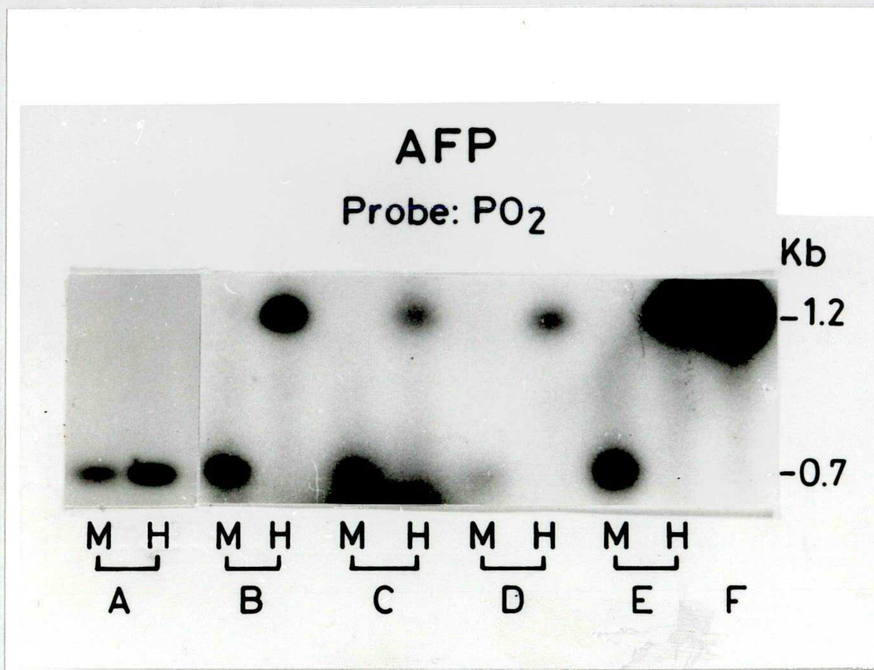

Fig. 13 . Methylation state of the 5' region of the rat AFP gene ,site M-2. Genomic DNA was digested as described in the legend of Fig.12. Hybridized with probe PO-2. Lane A. Fα6; B. VIII-3-23; C. VIII-2-12; D. VIII-4-29; E. H56; F. probe PO-2.

3.3.2 Methylation pattern of the 5'region of Alb gene

The principal question in the case of Alb gene is the methylation state of the M+1 site. This side is located about 800 bp downstream from the cap site in the first exon of the Alb gene and the methylation state of this site is correlated with the expression of the Alb gene (Tratner I. et al. Mol. Cell Biol. 7:1856-1864,1987).

Fig.14 shows the methylation state at the site M+1. In the *Fα* 6 Alb+ parental cell line, two bands of the same size approximately 1,0 kb can be observed when HpaII digestion was performed, the appearance of a band with similar M.W., as in the case of MspI digestion, signifies that the probed site is demethylated. Two bands corresponding to fragments with different size can be observed in MspI and HpaII digestions in the H56 Alb- parental cell line, as well as in the hybrid cell lines. The highest band corresponds to the EcoRI-HindIII fragment and signifies that the probed site is methylated.

Hybridization with the probe sub JA resulted in a complex pattern due to the presence of three MspI sites in that DNA region. Fig.15 shows the methylation pattern of site M-2 located about 3,5 kb from the cap site. The M-2 site is methylated even when the Alb+ parental cell line transcribes the albumin gene. The levels of methylation of these sites do not correlate with Alb gene expression.

The methylation state of the M+1 site is correlates with the Alb gene expression, is undermethylated in the Alb producer and methylated in the Alb nonproducer cell lines.

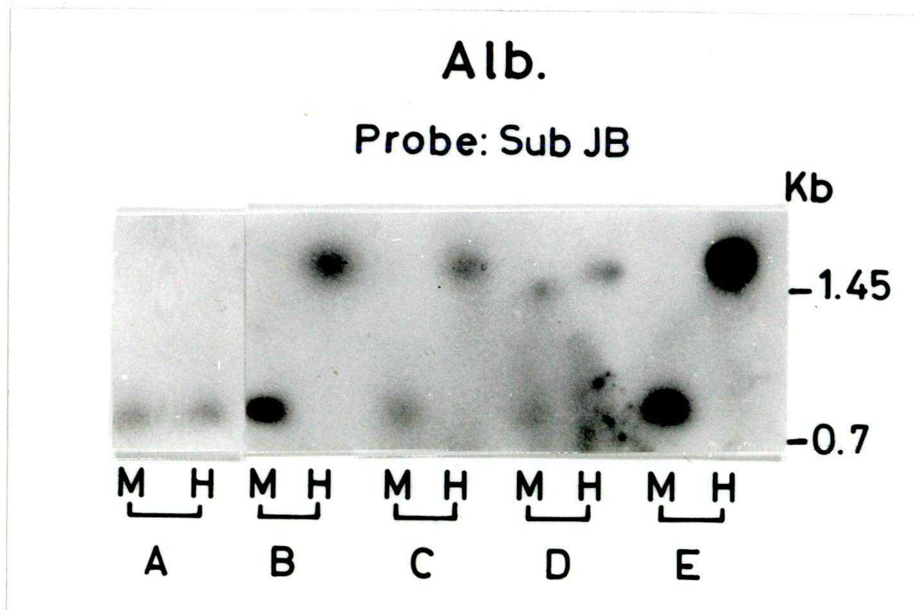

Fig. 14 . Methylation state of the 5' region of the rat Alb gene, site M+1. Genomic DNA was digested as described in the legend of Fig.12. Hybridized with probe Sub JB. lane A. Fα6; B. VIII-3-23; C. VIII-2-12; D. VIII-4-29; E. H56.

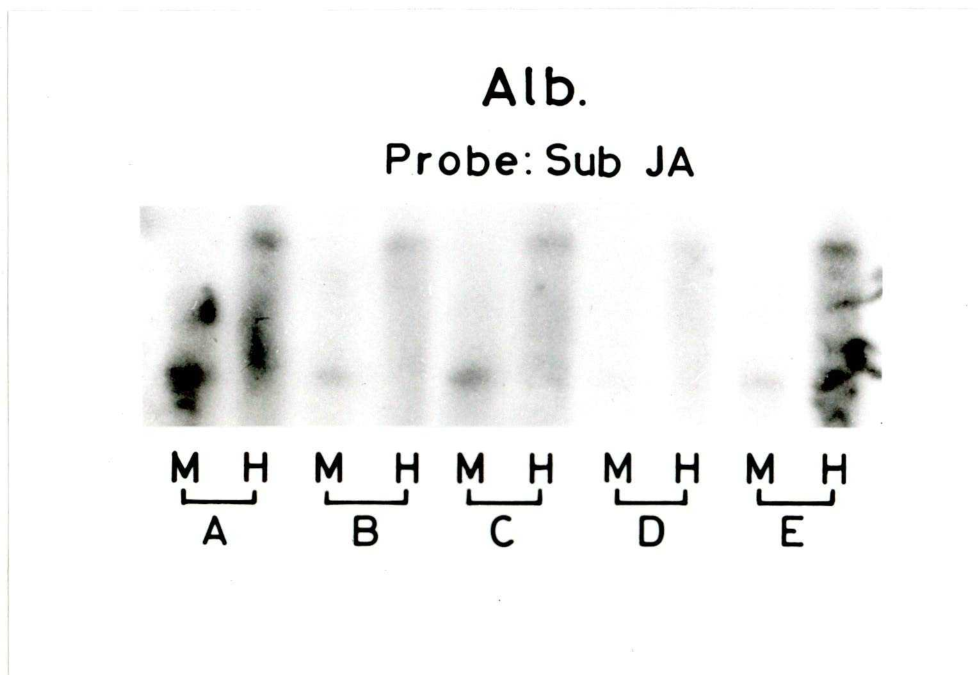

Fig. 15 . Methylation state of the 5'region of the rat Alb gene, site M-2. Genomic DNA was digested as described in the legend of Fig.12. Hybridized with probe Sub JA. Lane A. Fα6; B. VIII-3-23; C. VIII-2-12; D. VIII-4-29; E. H56.

3.3.3 Effect of 5-Azacytidine on the methylation pattern of some specific sites in the 5'region of AFP and Alb genes

As was previously described, no re-expression of AFP and Alb genes was found in 5-Azacytidine treated hybrid cell lines. Total genomic DNA/s were prepared from these treated cell lines and the methylation state of some specific sites in the 5'region of AFP and Alb genes were determined as was already described.

Fig.16 shows the methylation state of AFP site M 0 in a hybrid cell line treated with different concentration of 5-Azacytidine. No differences could be observed between the lane A. control untreated cell line and lanes B.-F. treated with different concentrations of 5-Azacytidine.

Similar results were obtained for the AFP site M-2(Fig.17)and for the Alb site M+1(Fig.18). No differences in the methylation state of these sites were observed between the untreated and 5-Azacytidine treated hybrid cell line.

These results are somewhat unexpected and at this moment they are difficult to interpret, but recently, it was shown that some methylation site were persistently methylated even after multiple consecutive 5-Aza. treatments(Michalowsky l. A. et al. Mol. Cell Biol.vol 9 :885-892,1989).

The results presented about the methylation pattern of the 5' region of the AFP and Alb genes are in good agreement with previously published results obtained in the AFP and Alb producer and non-producer cell lines and tissues (Ott M-O. et al. Cell 30 : 825-833 1982 and Tratner I. et al. Mol. Cell Biol. 7: 1856-1864 1987). However we do not know the exact function of

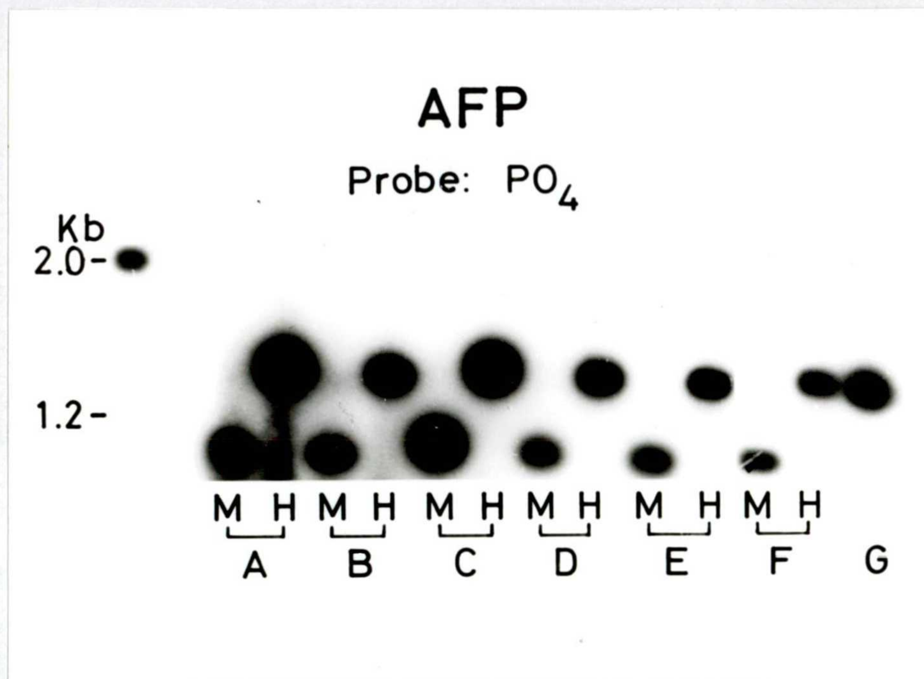

Fig. 16 . Methylation state of the AFP site M 0 in 5-Aza. treated hybrid cell line. Genomic DNA was digested as described in the legend of Fig.12. Hybridized with probe PO-4. Lane A. control, untreated hybrid cell line; B. 3 μM Aza. 0 hours; C. 6 μM Aza. 0 h.; D. 3 μM Aza. 24 h.; E. 6 μM Aza. 24 h.; F. 6μM Aza. 48 h.; G. PO-4 probe.

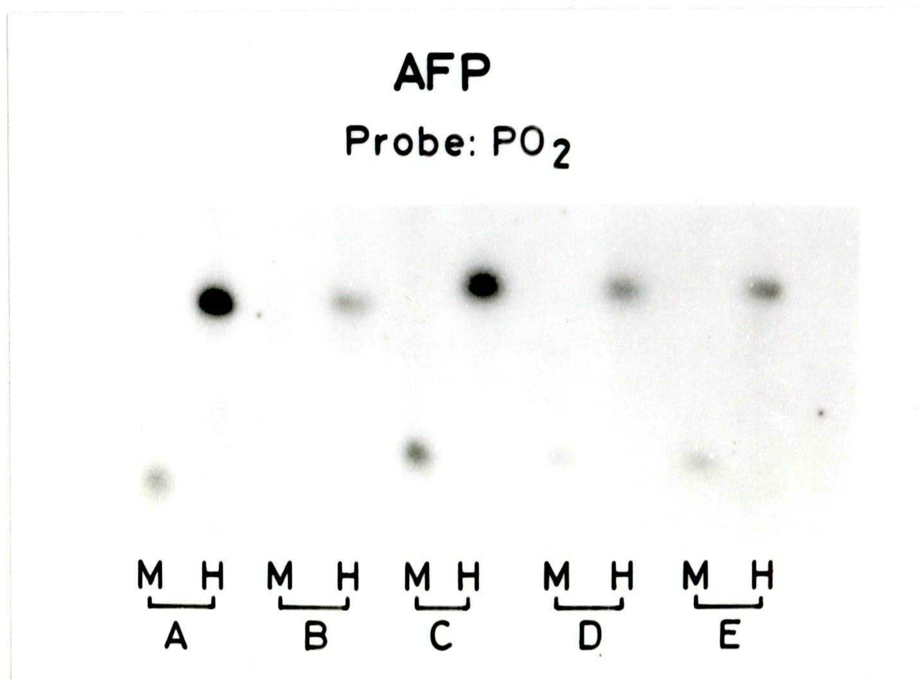

Fig. 17 . Methylation state of the AFP site M-2 in 5-Aza. treated hybrid cell line. Genomic DNA was digested as described in the legend of Fig.12. Hybridized with probe PO-2. Lane A. control, untreated hybrid cell line; B. 3 µM Aza. 0 h.; C. 6 µM Aza. 0 h.; D. 3 µM Aza. 24 h.; E. 6 µM Aza. 24 h..

SZTE Egyetemi Könyvtár SZEGED

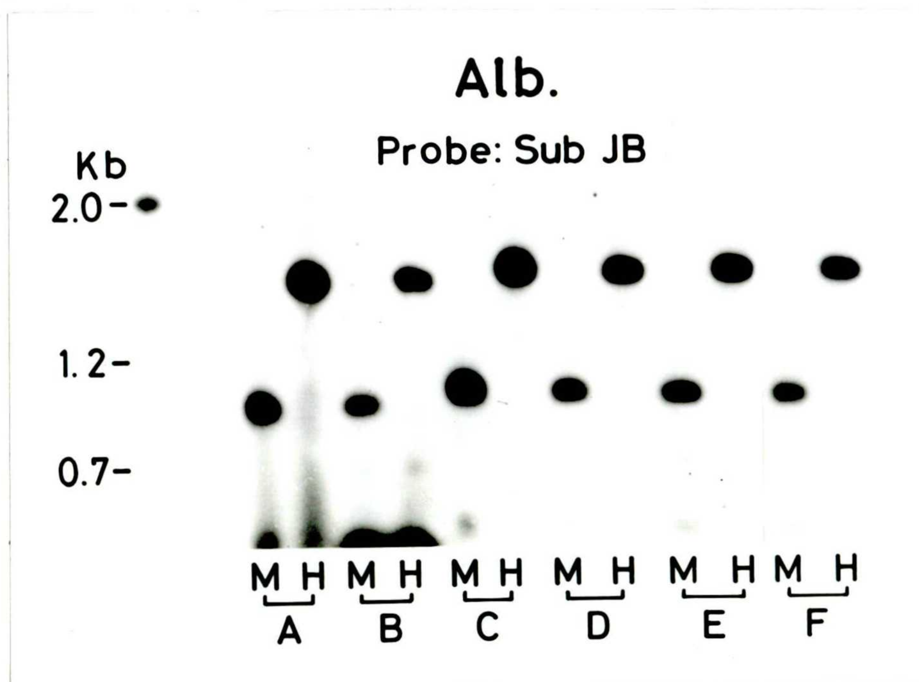

Fig. 18 . Methylation state of the Alb site M+1 in 5-Aza. treated hybrid cell line. Genomic DNA was digested as described in the legend of Fig.12. Hybridized with probe Sub JB. Lane A. control, untreated hybrid cell line; B. 3 μM Aza. 0 h.; C. 6 μM Aza. 0 h.; D. 3 μM Aza. 24 h.; E. 6 μM Aza. 24 h.; F. 6 μM Aza. 48 h..

DNA methylation, if the methylation is a cause or a consequence of extinction or if it acts only to stabilize the gene extinction. The methylation pattern of the AFP and Alb genes in the hybrid cells were similar to the AFP and Alb negative parental cell line. We presume that the mechanism of extinction of AFP and Alb genes in the hybrid cell lines is similar to the observed in the AFP and Alb negative parental cell line.

The regulatory significance of these sites is supported by their location near regions defined as important for AFP and Alb gene control. The AFP M 0 is located within the tissue-specific promoter (Muglia L. et al. Proc. Natl. Acad. Sci. USA 83 :7653-7657 1986), the M-2 site is positioned in a region defined as a tissue-specific enhancer, between -3,7 -3,3 kb (Watamabe K.et al Jour.Biol.Chem. 262: 4912-4818 1987).

Of particular interest is that these three MspI sites,are closely located to some DNase I HS sites, as was shown by Tratner I. and co-workers (Mol.Cell Biol. 7:1856-1864 1987). It is thus likely that the DNA methylation and specific alterations in chromatin structure, like DNase I HS, define and expose cis-acting regulatory elements in such a way that trans-acting factors could then bound to these cis-acting regulatory elements (Keshet I. et al. Cell 44:535-543 1986).

3.4 Regulation of TAT activity in different hepatoma cell line variants

As was already stated the data about regulation of TAT gene is contradictory. The main question is the level of regu-

lation of the basal and Dex induced TAT (transcriptional and/or translational level) in hepatoma cell line variants.

The TAT activity was determined in the hepatoma cell line variants and the results are presented in Fig.19 A. It can be seen that the Fao dx3 M have a relatively high basal 142,4 and induced 1217,0 TAT activity, the I/B value is 8,8. The second and third lines have 63,9; 91,3 as basal and respectively 137,0; 141 as induced TAT activity. The I/B value is 2,1 in the Fao dx3 F and 1,5 in the Fao dx3 O cell line.

Fig.19 B shows the Northern blot analysis of TAT mRNA sequences from total RNA preparations from paralell cell cultures of the same cell lines. The total RNA was prepared simultaneously with the determination of TAT activity. The signals corresponding to the 2,4 kb TAT mRNA and the 1,2 kb G3PDH mRNA, used as control, can be observed. No significant differences in the RNA quantity or quality, can be seen based in the fact that the signal intensity of the 1,2 kb G3PDH is almost the same in every line.

The analysis of the results clearly showed that the value of I/B 8,8 determinated by biochemical methods correspond to nine fold increase of the induced TAT mRNA in the Fao dx3 M cell line. However, the values of I/B determinated from the Fao dx3 F 2,1 and 1.5 from the Fao dx3 O cell lines, through determination of TAT activity do not correspond to the increase of the induced TAT mRNA. The increase of induced TAT mRNA is approximately six-fold, however the I/B values is 2,1 and respectively 1,5.

As was already stated the action of Dex., the glucocorti-

A

Cell line	T A T (a)		
	B (b)	I (b)	I/B
Fao dx3 M	142,4	1217,0	8,8
Fao dx3 O	91,3	141,0	1,5
Fao dx3 F	63,9	137,0	2,1

B

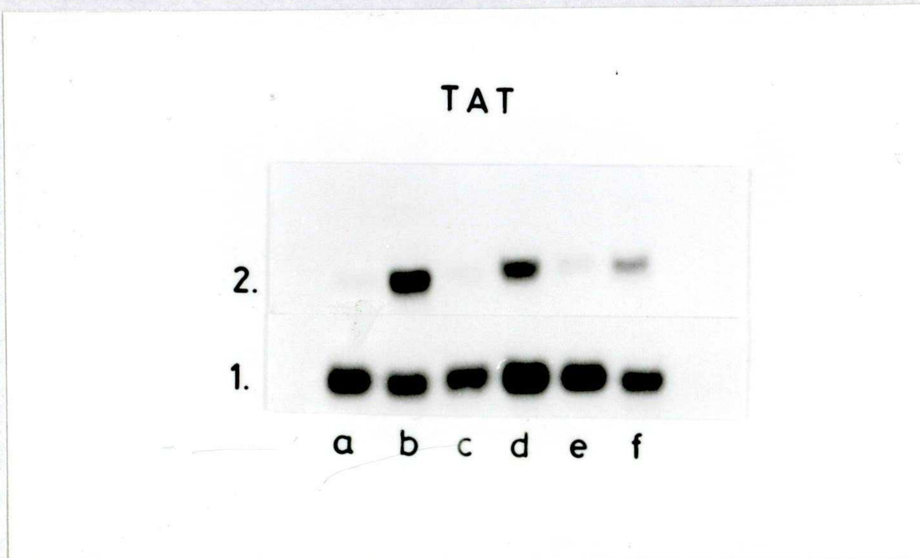

Fig. 19 A . Basal and induced TAT activity in hepatoma cell line variants.

(a) Specific Enzyme Activity expressed in mU/mg protein.

(b) the given value is the average of 3 measurements.

B. basal ; I. induced by $1 x 10^{-6}$ M Dex.

B . Northern blot analysis of the TAT mRNA in hepatoma cell line variants. 25 μg of total mRNA per lane was hybridised with TAT cDNA probe pc TAT-3. Lane a. Fao dx3 M basal ; b. induced ; c. Fao dx3 O basal ; d. induced ; e. Fao dx3 F basal; f. induced. 1. 1,2 kb G3PDH mRNA's, 2. 2,4 kb TAT mRNA's.

coid hormone used for TAT indution is mediated by the glucocorticoid hormone receptors. For this reason we analised the gluc. rec. mRNA in the previously mentioned cell lines and the results are presented in the Fig. 20. The signals corresponding to the 7.0 kb gluc. rec. mRNA and the 1,2 kb G3PDH mRNA can be seen.

As was expected no significant differences in gluc. rec. mRNA, non in the RNA quantity or quality can be observed.

As conclusions it can be affirmed that :

- the regulation of basal TAT is at transcriptional level
- there is no doubt that, the indution of TAT by Dex. is made at transcriptional level, however, two of the examined cell lines show a post transcriptional regulation, probably at the translational level.
- the content of gluc. rec. mRNA is the same in the three cell line variants as well as the level of the induced TAT mRNA in two of the examinated cell lines.

The regulation of TAT activity is principally at transcriptional level similar to the other examined hepato-specific functions, but there is doubtless evidence that in two of the examinated cell lines a post transcriptional regulation acts in the expression of induced TAT activity.

3.5 Complementation analysis between different glucocorticoid sensitive hepatoma cell lines

To investigate these Dex resistant glucocorticoid receptor minus and plus hepatoma cell lines with regard to complemen-

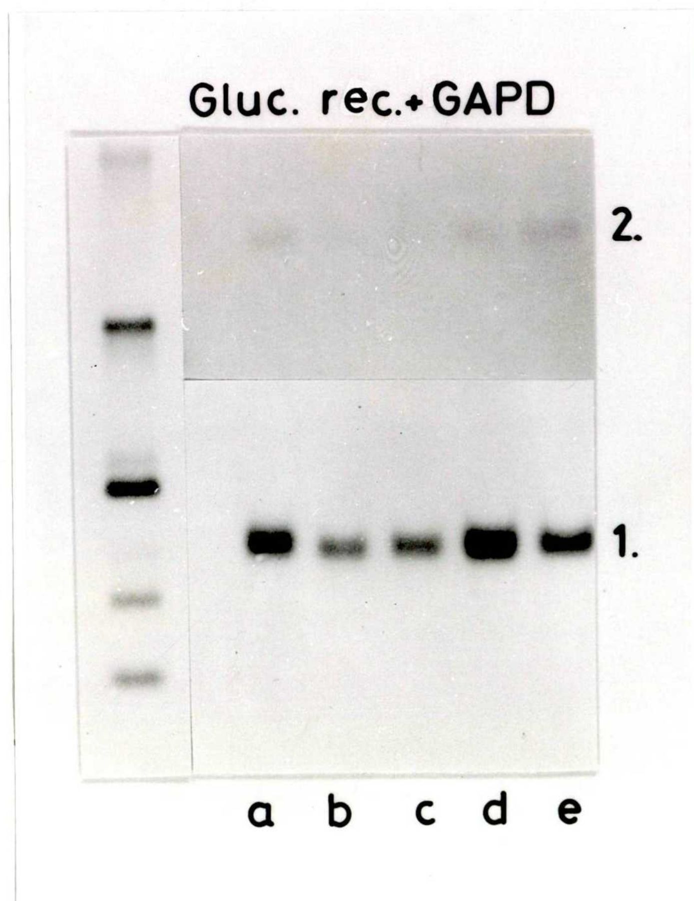

Fig. 20 . Northern blot analysis of the gluc.rec . mRNA in hepatoma cell lines with different TAT activity. 25 µg of total RNA were hybridised with gluc. rec . cDNA psG1, and G3PDH cDNA probe. Lane a. Fao dx3 M basal; b. induced; c. Fao dx3 F basal; d. induced; e. Faodx3 O basal. 1. 1,2 kb G3PDH mRNA's; 2. 7 kb gluc.rec . mRNA's.

tation of the lysis function (Dex sensitivity) in pair-wise crosses, an essential step is to carry out a test for the dominance or recessiveness of the phenotype in question.

In order to determine the dominante phenotype in hepatoma cell lines, a cell hybrid was made between Faza 967 R+, s, steroid-responsive phenotype and S-H56-125 R-, r, steroid--nonresponsive phenotype .

The results are presented in Tab. 3 . The three examined hybrid clones, from the previously mentioned fusion IX-4-1, IX-4-2, IX-5-5, were shown to be Dex sensitive by plating test, conferring the expected results that the Dex sensitive phenotype is dominant.

Confirming the absence of a diffusible factor, in the steroid-resistant cell line, the Dex resistant phenotype is recessive and the Faza 967 cell line contains biologically functionable receptors. The dominance of the glucocorticoid responsive phenotype was found by others authors also (Yuh Y-S. and Thompson E.B. Som.Cell Mol. Genet. vol.13: 33-46 1987).

The next step was to make an attempt to carry out complementation between different glucocorticoid-resistant hepatoma cell lines. Five different crosses were made between a Dex resistant R+, r Clone 2 cell line and five different Dex resistant R-, r S-H56 clones.

Clone 2 x S-H56-106

Clone 2 x S-H56-107

Clone 2 x S-H56-112

Clone 2 x S-H56-116

Clone 2 x S-H56-125

Tab.3. Characterization of paternal cell lines and cell clones obtained by fusions

Cell line	Time after fusion (days)	Colonies formation		Dex. sensi-tivity	Steroid receptor	Chromosome number	
		Size	Number			Range	Mean
Faza 967	38	s	i	s,i			
Clone 2	55	r	r	r,r	$5,4x10^3$	83-89	87
S-H56-106	76	r	r	r,r	a	40-54	50
S-H56-107	76	r	i	r,i	a	48-53	51
S-H56-112	-11	r	i	r,i	a	49-55	52
	68	r	r	r,r	a	49-55	52
S-H56-116	-18	r	r	r,r	a		
	40	r	i	r,i	a		
S-H56-125	75	r	i	r,i	a		
Hybrids S-H56-112 xFaza 967							
IX-4-1	20	s	s	s,s			
IX-4-2	20	s	s	s,s			
IX-5-5	25	s	s	s,s			
Clone 2 x S-H56-106							
I-1-1	77	r	r	r,r	$3,0x10^3$		
	148	r	r	r,r			
I-1-4	46	r	r	r,r		112-137	124
	50	r	r	r,r			
I-1-7	84	i	r	i,r			
I-3-18	76	r	r	r,r			
I-4-20	59	i	r	i,r			
Clone 2 x S-H56-107							
II-3-3	50	i	r	i,r		95-137	123
	84	i	r	i,r			
II-3-7	50	r	r	r,r			
II-1-11	62	r	i	r,i			
II-2-13	62	r	r	r,r			
	115	i	r	i,r			
II-2-14	76	i	r	i,r			
Clone 2 x S-H56-125							
III-3-5	54	r	i	r,i			
	98	r	r	r,r		124-136	131
	115	r	i	r,i			
	123	r	r	r,r			
III-4-7	51	r	r	r,r			
	75	r	r	r,r	$8,58x10^3$		
III-1-9	64	i	i	i,i			
	105	s	i	s,i	$8,50x10^3$		
	118	s	i	s,i			
	120	i	r	i,r			
III-1-13	98	i	r	i,r			
	114	s	i	s,i			
	116	i	i	i,i			

Continuation of Tab.3

Cell line	Time after fusion (days)	Colonies formation		Dex. sensi-tivity	Steroid receptor	Chromosome number	
		Size	Number			Range	Mean
III-4-19	76	r	r	r,r			
	98	r	r	r,r			
	129	r	r	r,r			
III-3-2	78	r	r	r,r			
	108	r	r	r,r			
	110	r	r	r,r			
III-3-1	78	s	s	s,s			
	93	s	s	s,s			
Clone 2 x S-H56-112							
V-5-4	49	r	r	r,r			
V-5-7	68	r	i	r,i			
V-5-8	74	r	i	r,i			
	91	r	i	r,i			
V-6-13	74	r	r	r,r			
V-6-16	68	i	r	i,r			
	128	r	r	r,r			
Clone 2 x S-H56-116							
VI-2-2	57	r	r	r,r			
VI-2-9	44	r	r	r,r			
VI-1-16	37	r	i	r,i			

(a) Less then 10-20% of the parental cell line H56 $2,94 \times 10^3$

From each cross approximately five independent clones were characterized for Dex sensitivity by plating test and the results are presented in the Tab. 3 , and Fig. 21 . The parental cell lines used in these crosses are Dex resistants Tab.3;Fig.21 A,B. Among four different crosses no Dex sensitive hybrid clones were identified Tab.3, Fig.21 C, D, however different degrees of Dex resistance were found, especially when the two parameters of plating test gives slightly different values.

Among the isolated hybrid clones from one of the crosses (Clone 2 x S-H56-125) sensitive and resistant phenotypes were found Tab.3,Fig.21 E. From the seven charaterized hybrid clones, three were found to be Dex sensitive and the others four Dex resistant.

As was shown by chromosome analysis the examined cell clones are cell hybrids obtained by the fusion of the parental cell lines Fig.22 . The III-1-4 cell hybrid clone contains the chromosomal set of the parental cell lines. As it can be observed in Tab. 3 a limited chromosome loss appear in the different clones (124, 123, 131) but the expected maximal chromosome number can also be found.

In order to investigate the glucocorticoid receptor in the parental and hybrid cell lines, the determination of the glucocorticoid receptor content and Northern blot analysis of glucocorticoid receptor m RNA was carried out.

The Clone 2 r, R+ cell line have $5,4x10^3$ receptors per cell, the S-H56 r R- cell lines have less than 10% of the parental cell line H56. Among the analysed cell hybrids no significant difference in glucocorticoid receptor content, that could

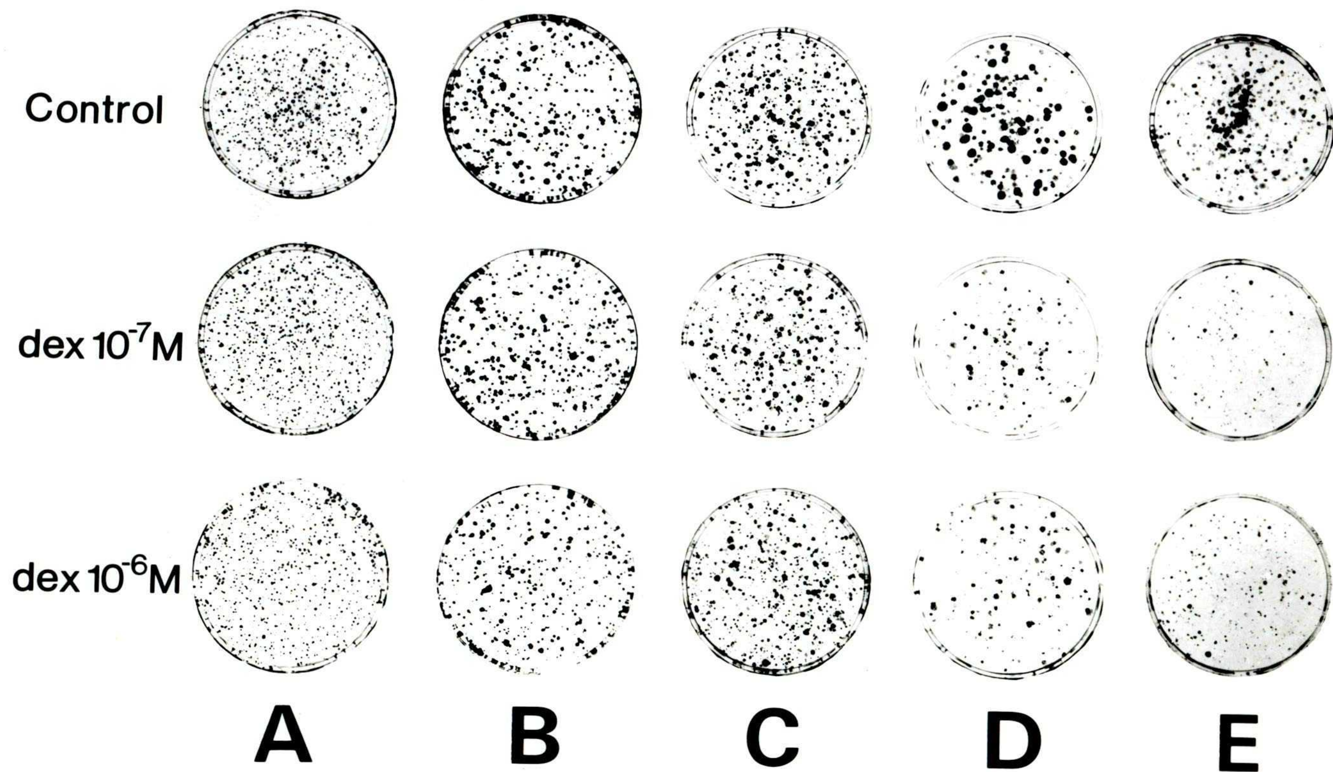

Fig. 21 . The effect of Dex on colony formation of parental and hybrid cell lines (see Materials and Methods for details). Panel A. Clone 2; B. S-H56-125; C. Clone 2 x S-H56-106 , I-1-7; D. Clone 2 x S-H56-107 , II-3-3; E. Clone 2 x S-H56-125 , III-1-9.

explain the different sensitive or resistant phenotype, was found by the techniques of steroid binding by whole cells.

The Northern blot analysis of the glucocorticoid receptor mRNA sequences from total RNA preparetions is shown in Fig.23.

It can be seen that only the receptor positive cell lines give a signal with an approximately molecular weight of 7 kb, and again there is no difference at receptor mRNA level that can explain the differences in phenotype.

In conclusion we have shown that :

- the glucocorticoid sensitive phenotype is dominant in hepatoma cell lines and the S-H56-112 cell line did not possess trans-dominant negative function that prevent growth inhibition.

- the glucocorticoid R- S-H56-125 cell line can be complemented by the R+ Clone 2 cell line, giving a glucocorticoid sensitive phenotype.

- the existence of more then two intervening genes in the growth inhibition; because in the complementation of the R- phenotype is not enough for the restauration of glucocorticoid sensitive phenotype.

- there is no reactivation of the receptor gene in the S-H56 clones.

- the observed glucocorticoid resistant or sensitive phenotype among the cell clones isolated from the same fusion and from different fusions cannot be explained by the differences in glucocorticoid receptor content either at receptor mRNA level.

This data is in agreement with the data presented by others, however, some questions remain to be answered.

There is no doubt, that two different types of glucocorticoid

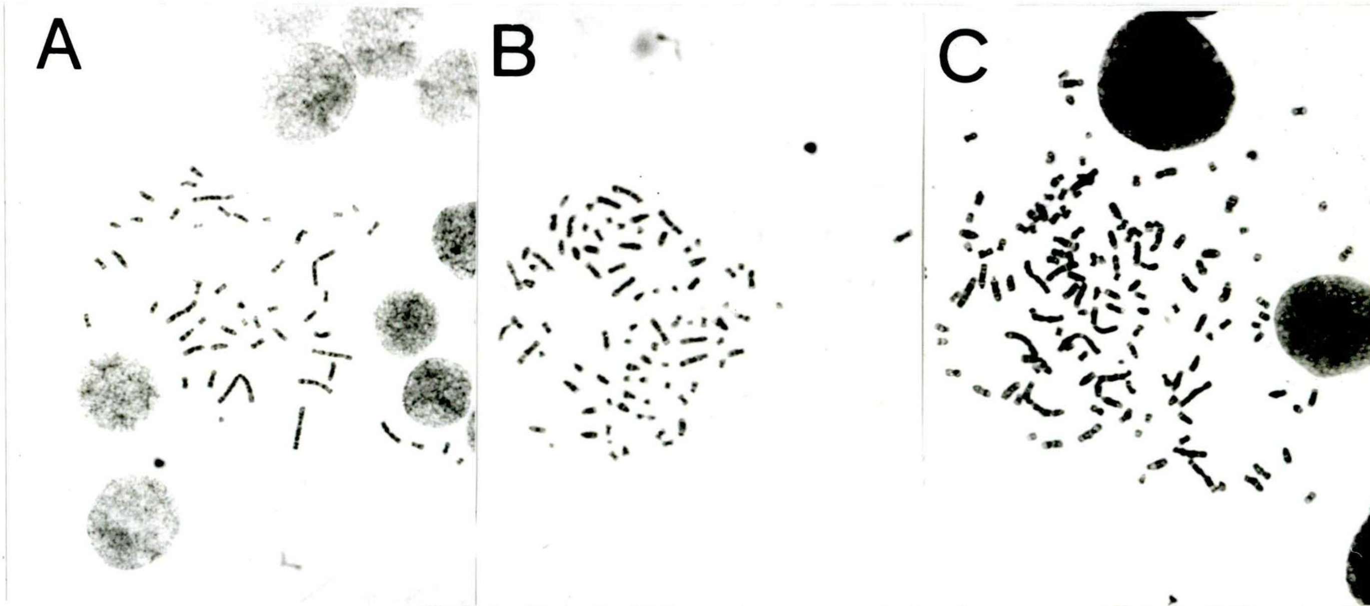

Fig. 22 . Mitosis from parental and hybrid cell lines.

A. S-H56-106 ; B. D12HbDex ; C. Clone 2 x S-H56-106, I-4-7.

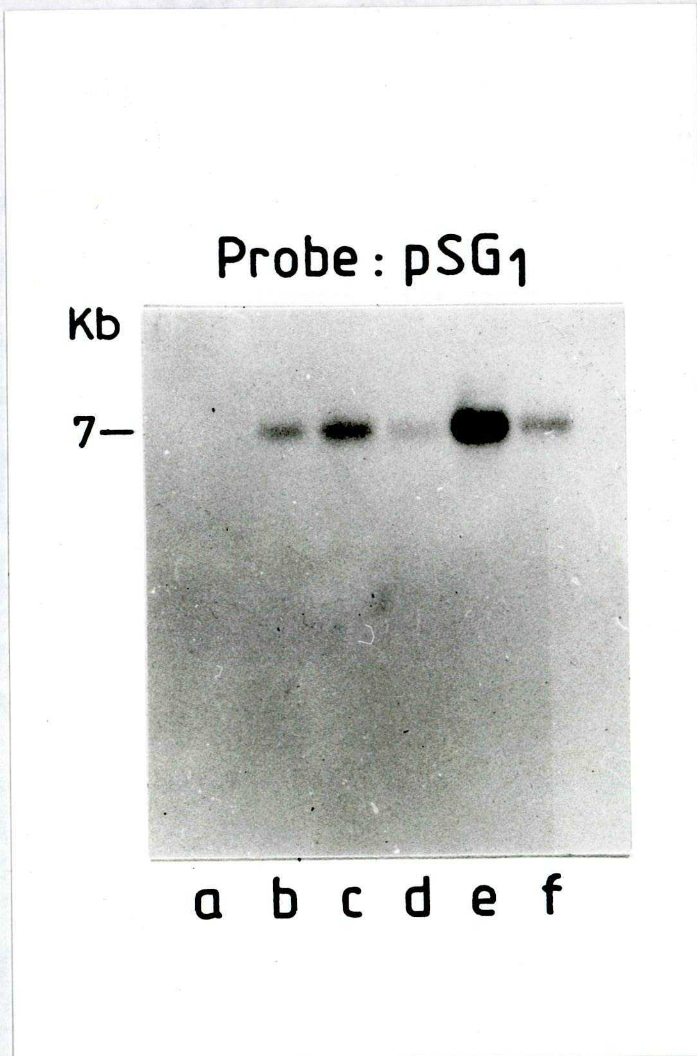

Fig. 23. Northern blot analysis of gluc. rec. mRNA in parental and hybrid cell lines. a. S-H56-126; b. III-1-9; c. III-4-7; d. I-1-1; e Faza 967; f. H56.

receptor mRNA were observed in cells of different species and organs and these differ in the length of their untranslated 3' sequences. However, the available data about the molecular weight and function of this mRNA types are confusing. Miesfeld R. and co-workers (Nature 312 :779-781 1984) affirm that the glucocorticoid receptor transcript is 6 kb, but the 6 kb mRNA in r- and the 5 kb mRNA in the nt' mutants may encode nonfunctional 94 Kd and 40 Kd proteins.

In 1986 Miesfeld R. and co-workers (Cell 46 :389-399 1986) affirm that the two types of glucocorticoid receptor mRNA are of 6,5 kb and 4,8 kb.

Kalinyak J.E. et al. (Jour.Biol.Chem. 262 :10441-10444 1987) and Gehring U. et al (Mol.Cell Endoc. 48: 89-96 1986) confirm a 7 kb major mRNA and a 5 kb minor mRNA, and in variants of the nt' phenotype a major 5,5 kb receptor mRNA from which 5' sequences are missing.

The molecular weight of glucocorticoid receptor mRNA observed by us is 7 kb in both parental and hybrid cell lines. The same molecular weight mRNA was observed in cell lines with normal TAT activity.

It can be affirmed that this 7 kb molecular weight glucocorticoid receptor mRNA found by us in different hepatoma cell lines with common origin is biologically functionable.

A second question is how to explain the different sensitive and resistant phenotypes observed among the isolated clones from the same fusion.

It seems possible that these different phenotypes can be caused by different chromosomal events like :chromosome loss, etc.

However, the further characterization of this event is almost impossible, taking in acount the high chromosome instability and the mean chromosome number observed.

Such loss of certain chromosomes was already observed to be responsible for the alteration of the observed phenotype (Gal A. and Venetianer A. Cytog.Cell Genet. 35:75-77 1983). Similar to them we observed a marked size heterogenity among the colonies in the presence of different Dex concentration.

Little information about the detailed process of growth inhibition is available. It seems that the growth inhibition is a complex cascade event, where several genes are responsible for this, at least more then two, being a multistep function.

It seems that the S-H56 clones contains different defects in this multistep genes, that S-H56-106; 107; 116; 112; are Gr $^{a- b- c+}$ R-, (Gr^{a,b,c,} are different genes responsable for the growth inhibition function) but the S-H56-125 is Gr $^{a+ b+ c-}$R-, the other parental cell Clone 2 is Gr $^{a+ b- c+}$R+. From the fusion Clone 2 x S-H56-106 107, 112, 116 arose a glucocorticoid resistant phenotype Gr $^{a+ b- c+}$ R+ and the glucocorticoid sensitive phenotype Clone 2 x S-H56-125 Gr $^{a+ b+ c+}$R+.

This means that the glucocorticoid receptor is complemented in all crosses, but the growth (Gr) inhibition function is complemented only in some hybrid clones from Clone 2 x S-H56-125 cross.

3.6 General discussion

There is no doubt about the importance of trans-acting factors in gene regulation, in temporal and selective activation or repression of particular genes during the differentiation (Maniatis T. et al. Science vol236 :1237-1245, 1988). In the case of several hepato-specific genes, it was demonstrated the existence of regulatory genes, acting through trans-acting factors by binding to cis-acting regulatory regions.

The presence of such regulatory genes were demonstrated in the case of AFP; in mouse liver the adult basal level of mRNA is determined by the raf gene (Olssan M. et al. Jour. Exp. Med. 145: 819-827 1977) and the magnitude of the transient induction of AFP mRNA during regeneration by a gene termed rif (Belayew A. et al. Mol.Cell.Biol. 2: 1427-1435 1982).

In the case of TAT, a control region is required for the mouse TAT gene expression has been assigned near to the albino locus on chromosome 7 (Schmid W. et al. Proc. Natl. Acad. Sci. USA 82: 2866-2869 1985).

In the case of LDH, the existence of two regulatory genes has been found in mouse, the Ldh-1 and Ldh-2, as was mentioned in the introduction.

The existence and envolvement of such trans-acting factors in the regulation of genes coding for differentiated functions have been shown, also by somatic cell hybridization experiments, as in fusion of fibroblast cells with hepatoma cells. These factors are positive or negative regulatory factors. Another type of differentiation specific factors were also identified, as the

Albumin Proximal Factor (APF), a promoter specific transcriptional factor identified by Cereghini S. and co-workers. Homologous sequences to the APF recognition site were found in the promoter region of other liver specific genes (Cereghini S. et al. Genes Develop. 2:957-974 1988).

Such differentiated hepato-specific functions are regulated in a co-ordinated manner. This is also supported by the fact that differentiation and reversion of hepatoma cell lines affect the entire group of hepato-specific functions. In this study, one of the conclusions, was the total, stable and co-ordinated extinction of the examinated hepato-specific functions. This supposes that the H56 dedifferentiated parental cell line possess a trans-acting factor or factors that leads to the extinction of these hepato-specific functions. Such factors were called tissue-specific extinguishers (tse's) and are responsible for the extinction of TAT and Alb in liver fibroblast hybrids (Killary A.M. et al. Cell 38:523 1984;Petit C. et al. Proc.Natl. Acad.Sci.USA 83:2561 1986). Two different acting mechanisms of these factors can be supposed. First, the trans-acting factors from the H56 cell line are acting on the regulatory genes and the second possibility that these trans-acting factors are acting directly on the structure genes. The first possibility seems the most probable, on the basis of actual knowledge. However, there are several possible explanations for how this could occur, but this is not our main object. One of the possibilities is that the common differentiation regulatory factor prevents the expression of several putative positive regulatory trans-acting factors. Another possibility is that

these factors switch on a negative trans-acting regulatory factors and these prevent the binding of the positive regulatory factors, or by protein-protein interaction, preventing the transcription.

In the case of the rat growth hormone (rGH) the combination of positive and negative regulatory elements contributes to the extinction of rGH in fibroblast, pituitary cell hybrids. A pituitary-specific protein is absent and a negative regulatory element is activated (Tripputi P. et al. Science vol.241:1205-1207 1988).

Whatever the exact mechanism of the interaction of the trans-acting factors and regulatory genes, and the trans-acting factors and structure genes are, it seems probable that several differentiation regulatory genes regulate, in fact, the differentiation state of a given cell, by acting on a group of regulatory genes. One or more of such regulatory gene regulates the expression of a differentiated function, by swiching on or off the transcription of a gene. These regulatory mechanisms acting at transcriptional level, are integrated into the overall pattern of gene regulation during development.

The normal function of these mechanisms needs other regulatory elements such as the DNA motifs, in the 5'region of genes, necessary for the transcription; promoter elements, cis-acting elements, like cis-acting, cell type specific negative regulatory element, hormone responsive elements like GRE in our study, to mediate the increase transcription of TAT gene by Dex.

The transcriptional regulation is only one of the regulatory mechanisms. Over this is the post-transcriptional or translatio-

nal regulation. In the present study, such post-transcriptional mechanism regulates the induction of TAT activity by Dex in two of the examinated cell lines. Such post-transcriptional regulation was already stated by Wicks W.D. and co-workers (see Tirosine Aminotransferase).

In several studies the presence of trans-acting factors was shown not to be sufficient for their interaction with the DNA. In the case of TAT, Becker P.B. and co-workers (Cell 51:435-443 1987) showned that the presence of the factors in nuclei is not suficient for their interaction with potential target sites. It seems necessary or the modification of the proteins or a DNA signal to regulate the normal binding and protein DNA interaction. This signal can be a DNA modification like DNA methylation and indeed this can regulate the protein DNA binding and in this way the gene expression.

In several studies, the function of DNA methylation in the gene regulation was demonstrated. The correlation between the demethylated state of certain sites in the 5'region of genes and the transcription of this gene (Tratner J. et al. Mol.Cell.Biol. 7: 1856-1864 1987 , Razin A. Bioch. et Bioph. Acta 782: 331-342 1984). Another conclusion of the present study was the correlation between the expression of AFP and Alb genes and the hypomethylated stage of M 0 and M-2 respectively M+1 sites in the 5' region of this two genes (see Results and Discussion).

In other studies, as in that by Ott M-O. and co-workers(Cell 30: 825-833 1982) it was demonstrated that the DNA methylation is necessary but not sufficient for the gene expression.

In the overall mechanism of gene regulation the presence of

trans-acting factors is necessary a given pattern of methylation of the specific CG dinucleotides, as well as the specific alteration of the chromatin structure for the protein-DNA interaction and the consequent regulation of gene expression.

Several doubts still exist but we think that the present study can contribute to the understanding of the regulation of gene expression in hepatoma cell lines.

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