GENETIC ANALYSIS OF SMAD2 GENE IN HEPATOCELLULAR CARCINOMA

A THESIS SUBMITTED TO
THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS
AND THE INSTITUTE OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE

By
ALPER ROMANO
JULY, 1998

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T Ü M

SEVDİKLERİME

Alper Romano

I certify that I read this thesis and that in my opinion it is fully adequate, in scope and in quality, as thesis for the degree of Master of Science.

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ABSTRACT

Genetic Analysis of Smad2 gene in Hepatocellular Carcinoma

Alper Romano

M.S. in Molecular Biology and Genetics

Supervisor: Dr. Cengiz Yakıcıer

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Hepatocellular carcinoma is one of the most malignant cancers and is the most frequent one in some regions in the world. Although it is a multistage disease, its genetic composition is not well understood. TGFβ is shown to be a strong inhibitor of cell growth and during hepatocellular carcinogenesis there is an escape from the anti-proliferative effect of TGFβ. Smad2 protein is the mediator of response to TGFβ and its gene is mutated in several cancers. To clarify the role of Smad2 in TGFβ signalling in hepatocellular carcinoma we performed single-strand-conformation-polymorphism (SSCP) analysis in five exons of Smad2 for 35 tumor samples and in C-terminal region for five hepatoma cell lines. Two alterations were found out of 35 samples and no abnormal expression or big deletions were observed in cell lines. Thus Smad2 might be involved at least a part of hepatocellular carcinomas.

ÖZET

Hepatoselüler karsinomlarda Smad2 geninin genetik analizi

Alper Romano
Moleküler Biyoloji ve Genetik Bölümü Yüksek Lisansı
Tez Danışmanı: Dr. Cengiz Yakıcıer
Temmuz 1998, 58 sayfa

Hepatoselüler karsinom en kötü huylu kanserlerden biri olup, dünyanın bazı yörelerinde en sık rastlanan kanser türüdür. Çok aşamalı bir hastalık olmasına rağmen genetik içeriği pek anlaşılamamıştır. TGFβ, karaciğer hücre büyümesinin güçlü bir engelleyicisi olup, hepatoselüler karsinom sırasında TGFβ'nın anti-proliferatif etkisinden bir kaçış söz konusudur. Smad2, TGFβ sinyalinin hücre dışından hücre çekirdiğine taşınmasından sorumlu bir proteindir ve bu genin bazı kanserlerde mute olduğu gösterilmiştir. Smad2 geninin hepatoselüler karsinomlardaki TGFβ sinyalindeki rolünü açığa kavuşturmak için beş exon için 35 tümör örneğinde ve C-ucu bölgesi için beş hepatom hücre hattında (cell lines) SSCP analizi uyguladık. 35 örnekte iki değişiklik bulundu ve hücre hatlarında ifade bozukluğu ya da büyük delesyonlar gözlemlenmedi. Sonuç olarak, Smad2, hepatoselüler karsinomların en azından bir kısmında rol oynuyor olabilir.

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ABBREVATIONS

APS	ammonium persulfate
bisacrylamide	N, N, methylene bis-acrylamide
bp	base pairs
cdk	cyclin dependent kinase
cDNA	complementary deoxynucleic acid
C-terminal	carboxy terminal
DCC	Deleted in Colorectal Cancer
DGGE	denaturing gradient gel electrophoresis
DPC4	Deleted in Pancreatic Cancer 4
ddH_20	deionized (MilliQ) water
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
dpp	decapentaplegic
EDTA	ethylene diamine tetra acetic acid
EtBr	ethidium bromide
g	gram
HCC	Hepatocellular Carcinoma
HBV	Hepatitis type B virus
HCV	Hepatitis type C virus
HNPCC	Hereditary Nonpolyposis Colorectal Cancer
kDa	kilodalton
LOH	loss of heterozygosity
µC	microCurie
Mad	Mothers-against-decapentaplegic
$MgCl_2$	Magnesium Chloride
µg	microgram
MH	Mad homolog
min	minutes
ml	milliliter
mM	millimolar
µl	microliter

MMTV	mouse mammary tumour virus
MSI	microsatellite instability
N-terminal	amino terminal
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RNA	ribonucleic acid
SCC	squamous cell carcinoma
sec	seconds
Smad	Sma- and Mad related gene
SSCP	single strand conformation polymorphism
TBE	tris-boric acid-EDTA
TEMED	N,N,N,N- tetramethyl- 1,2 diaminoethane
TGFβ	Transforming growth factor β
TβR	Transforming growth factor β receptor
Tris	tris (hydroxymethyl)-methylamine
UV	ultraviolet

1. INTRODUCTION

The liver is a large digestive gland where nutrients are processed. It comprises a mixture of cell types, although most prevelant cells are the epithelial liver cells , so called hepatocytes. Hepatocytes perform a broad range of metabolic and secretory tasks. Even though hepatocytes derive from gut epithelium, they have a different life-style compared to other gut epithelial cells. Under normal physiological conditions, adult hepatocytes are non-dividing cells but they retain their capacity to proliferate. Proliferation is seen only either as a response to massive hepatocyte loss (partial hepatectomy, hepatotoxic agent intake, acute and chronic hepatitis, cirrhosis) or as a result of loss of antiproliferative control (hepatocellular carcinoma) (Ozturk 1994). Therefore, cell proliferation plays a key rol during tumorigenesis, as observed in the rat model (Grisham 1996).

1.1 Hepatocellular Carcinoma

More than 80 percent of primary liver cancers are primary hepatocellular carcinoma (HCC) which derive from hepatocytes. It is the 7th most common cancer in males and males develop HCC 2-3 times more than women. HCC carries a poor prognosis, with survival times from diagnosis measured in months. Although surgical resection by partial or total hepatectomy is available, partial hepatectomy is associated with a very high recurrence rate (25 percent per year) (Isselbacher et al. 1991). HCC is the 4th most common cause of death from cancer, responsible for 300000 deaths each year.

While hepatitis B and hepatitis C viral infections are considered major etiological factors, poor nutrition, alcohol and cigarette consumption, food and water contaminants, natural plant toxicants and oral contraceptives may also play a role. The incidence of HCC is generally low in Western countries (1-7/100000) and high in South-east Asia and sub-Saharan Africa (25-75/100000) (Bosch and Munoz 1990).

1.1.1 Hepatic cirrhosis and fibrosis

The most frequent form of liver injury (hepatocyte destruction) occurs during chronic HBV and HCV infections. These infections cause decades of inflammation that ends up with fibrosis. Hepatic fibrosis is a response to liver injury by recruitment of inflammatory cells, activation of mesenchymal (stellate) cells and release of cytokines. An important feature of hepatic fibrosis is that it is a reversible accumulation of extracellular matrix in response to chronic injury in which nodules have not yet developed, whereas cirrhosis implies an irreversible process in which thick bands fully encircle the parenchyma, forming regenerating nodules (Burt 1993).

80% of HCC develop a cirrhotic liver, mostly due to chronic viral infections. Furthermore, about 38 percent of patients with HBV- associated cirrhosis had HCC at autopsy. It should be noted that although presence of cirrhosis increases the risk for HCC it is not a requirement (Craig et al. 1990).

1.1.2 HBV and HCV

HCV infection is a major risk factor for the development of HCC worldwide. Two facts are employed as evidence : 1) 50- 75 percent of HCC patients have anti-HCV or HCV RNA detectable in serum in southern Europe and Japan. 2) In patients with chronic HCV infection, progression can be noted from milder forms of hepatitis to cirrhosis, and eventually, to HCC. Unlike the HBV, HCV is not a DNA virus and doesn't become integrated into host genome. It is likely that HCV has an indirect effect by causing liver injury. After 20 years of infection, 6-8 percent of patients with chronic hepatitis C can be expected to have developed HCC (Di Bisceglie 1998).

Hepatitis B surface antigen is common in countries where HCC is also common. HBsAg carriers are 40-100 times higher at risk to develop HCC than the non-carriers. HBV is a DNA virus and after infection it becomes integrated into host genome. But the location of integration was shown to be random and probably has no effect on pathogenity. HBV doesn't possess any gene product with transforming activity. Its X gene product which acts as transcription factor both for the virus and the host genome, was demonstrated to bind to p53 and cause its inactivation. Thus, this can be a mechanism of tumour progression (Feitelson 1993, Ueda et al. 1995).

1.1.3 Aflatoxin

Aflatoxin is a myotoxin produced by some species of Aspargillus. In the regions were HCC is epidemic, aflatoxin is taken in as a food contaminant. It is the only etiological factor that was shown directly to cause p53 mutation. It targets the codon 249 of p53

genes, and impairs its activity. This mutation is a hotspot where aflatoxin exposure is high but low in other areas (Ozturk et al. 1991, Ozturk et al. 1994)

1.1.4 Genetics of HCC

Like many other cancer types HCC is a multistage disease while taking decades to develop it. But the genes which are inactivated during tumourigenesis are poorly identified.

It is known that overexpression and/or activation of cellular protooncogenes and deletion or inactivation of tumour suppressor genes occur during multi-step carcinogenesis through either point mutations, gene amplification, gene rearrangement, alteration in gene methylation pattern, or a change in transcriptional regulation. These genes encode proteins of the cellular signal transduction system, which function to regulate gene expression, cellular growth and differentiation (Alberts et al. 1994).

1.1.4.1 Allelotyping studies

Different LOH studies revealed that some chromosome regions are commonly deleted in HCC and these regions might harbour one or more tumour suppressor genes. Previously LOH were reported on 1p, 4q, 5q, 6q, 8p, 10q, 11p, 16p, 16q and 22q. An extensive allelotypic analysis of HCC let us identify new regions that were previously not detected (Nagai et al. 1997). The highest rates of LOH are shown in table 1.

Table 1 High Frequence of Loss of Heterozygosity (LOH) in HCC

Chr. Location	LOH rate (%)	Candidate Genes
2q36-37	29%	
4q35	40%	
6q27	36%	M6P/IGF2R
7p15	30%	
8p23	42%	
13q12-q13	30-32%	RB, BRCA2
16q23-q24	28%	
17p13	33%	p53

Other regions which were shown also to be deleted for the first time are in table 2.

Table 2 Other chromosomal regions lost during HCC

Chr. Location	LOH rate (%)
1q22-q23	20%
1q42-q43	24%
7q33-q34	20%
8q23-q24	23%
9p12-p14	21%
9q34-qter	20%
14q32	23%
17q24	21%

1.1.4.2 Mutations

During HCC, candidate genes are not commonly inactivated. Most of the tumor suppressor genes are rarely mutated. Additionally, mutations in oncogenes are also not common (table 3). Some alterations which change the expression or the activity of the protein have been observed in HCC (table 4).

Table 3: Mutations in tumoursuppressors and oncogenes

Genes	LOH%	Mutation	References
P53	51%	30-55%	Puisieux and Ozturk 1997
M6P/IGF2R	70% 61%	25% 55%	De Souza et al. 1995 Yamada et al. 1997
P16	 % 41 yes	4/26 germline 1/23 3/62	Chaubert et al. 1997 Biden et al. 1997 Kita et al. 1996
RB		Rare	Zhang X. et al. 1994
BRCA2		1/60 somatic 2/60 germline	Katagiri et al. 1996
DLC-1	44%		Yuan et al. 1998
PRLTS		2/48	Fujiwara et al. 1995
Ras		Rare	Shen and Ong 1996

1.1.4.3 Other alterations

Table 4: Alterations other than mutations in tumour suppressors and oncogenes in HCC

p16	48% de novo meth.	Chaubert et al. 1997
IGF2	20% overexpression	Li et al. 1997 Uchida K. et al. 1997
Cyclin D1	4/30 amplifications	Zhang Y. J. et al. 1993
p53	inactivation by HBx	Feitelson et al. 1993

1.2 Transforming growth factor β (TGFβ)

1.2.1 Biological effects of TGFβ

TGFβ is a 25 kDa homodimeric peptide which is produced by nonparenchymal liver cells and secreted in a latent form. It appears to be an important cytokine both in normal and diseased liver. Because it is able to increase the levels of many extracellular proteins, it has been closely associated with the promotion of fibrosis and progression of cirrhosis. In normal human hepatocytes no TGFβ messenger or protein is detectable. In cirrhotic livers although hepatocytes in nodules continue not to express TGFβ, some cells in fibrous septa expressed it. In hepatocellular carcinomas, clusters of tumoural hepatocytes express large amounts of TGFβ. Interestingly normal and cirrhotic liver cells express the TGFβ receptors but they disappear in plasma membrane of tumour hepatocytes though they were in cytoplasm (Bedossa et al.1995). The apparent paradox of how hepatocytes proliferate despite of production of elevated TGFβ production by nonparenchymal cells is best clarified in a rat model. Accordingly, TGFβ1 is expressed only in nonparenchymal cells, but TGFβ receptors were expressed in both cell types. As the liver regeneration reach its peak, TGFβ1 mRNA levels are elevated and there is a significant depression of TGFβ receptor mRNA levels only in hepatocytes (Date et al. 1998).

Transforming growth factors (TGF-β) are potent inhibitors of proliferation of most cell types in culture and in vivo. However many tumorigenic cell lines have lost response to negative growth-regulatory effects of TGFβ1 which is the most commonly studied TGFβ molecule. Thus, it has been thought that the understanding of the

molecular mechanism of the TGFβ will reveal much about the tumorigenesis. Although many cell cycle proteins have been thought as potential targets of TGFβ such as cyclins, cyclin dependent kinases (Cdks) and Cdk inhibitors, it is not clear yet whether these targets are directly involved or the effect on these targets are simply a consequence of the TGFβ1 induced growth arrest (Alexandrow and Harold 1995).

There has been conflicting results about the role of TGFβ1 at early and late stages of carcinogenesis due to in vitro studies and different approaches. Accordingly, TGFβ1 acts either as a tumour promoter or as a suppressor at early stages and either stimulates or suppresses the malignant progression during the later stages (Cui et al. 1996).

1.2.2 TGFβ signalling pathway

Although transforming growth factor β (TGFβ) was discovered as a peptide which stimulates the proliferation of rat kidney fibroblasts, it acts as a potent growth inhibitor of many normal cell types, including epithelial cells. It has been shown that it also induces cell differentiation and production of extracellular matrix proteins. TGFβ belongs to a large superfamily of peptides which are structurally conserved throughout the evolution and includes members such as activins and bone morphogenic factors (BMPs) and they play important roles in differentiation and morphogenesis. TGFβ has three different isoforms in humans (TGFβ1-3), encoded by different genes (Hoodless and Wrana 1994). It is secreted in a latent form from the cells and they become active by proteolysis in which IGF2R plays an important role (De Souza et al. 1995).

1.2.2.1 TGFβ receptors

It has now been firmly established that most of these factors, including TGFβ, signal through type I and II serine/threonine kinase receptors. Another receptor type called TβR-III is a proteoglycan with a short cytoplasmic domain. It is not required for the biological activity of TGFβ1 and TGFβ3 but may have an accessory function for TGFβ2. Type II and type I receptors are the main mediators of the TGFβ signalling, they contain a cytoplasmic kinase domain and extracellular cystein-rich regions. In addition, type I receptor has a highly conserved cytoplasmic glycine- and serine-rich segment (GS domain). In the absence of ligand, TβR-I and TβR-II exist as homomers in the cell surface. In addition, TβR-II is constitutively phosphorylated by cellular kinases and by itself (Derynck 1994). Upon ligand binding by TβR-II, TβR-I which alone is not able to recognize free TGFβ is recruited to form a heterotetrameric terniary complex. Then, TβR-I is phosphorylated by the kinase activity of TβR-II at its threonine and serine residues of GS domain. TβR-II itself is not a substrate of TβR-I and its phosphorylation is not altered as a consequence of ligand-induced complex formation. Thus, it can be concluded that TβR-I is downstream of TβR-II in the TGFβ signalling and its kinase activity is essential for the phosphorylation of downstream substrates.(Wrana J. L. et al. 1994) (for figure see Appendix).

1.2.2.2 Smad proteins

The receptors for TGFβ are quite specific and no other ligand has been shown capable of binding to them. But it is also clear that identification of downstream targets of TβR-I would reveal much information about the specific effects of TGFβ. Smad proteins were identified as human homologues of Mad protein which mediates TGFβ -like dpp signalling in Drosophila. At present, at least eight distinct Smad proteins were identified in humans. They are highly conserved at their amino-terminal MH1 and carboxy-terminal MH2 domain among themselves and other vertebrates. These two domains are connected by a linker region which is less well conserved. It is extremely rich in serine and proline residues. Structural analysis showed that no known structural motifs exists in these genes. However sequence comparisons have revealed that Smad1, Smad5 are highly homologous and form a group. Indeed functional and biochemical analysis have proved that they specifically carry BMP signalling while Smad2 and Smad3 form another group and are involved in activin/TGFβ signalling. Thus, different signals recruit distinct Smads. These Smads are called receptor-regulated Smads. Smad4 is more distantly related to the other Smads and is a central protein for both pathways. Smad6 and Smad7 thought to act as inhibitors of these signals (Kretzschmar and Massague 1998). It has been established that the MH2 domain of receptor-regulated Smads is responsible for most of its functions. The function of MH1 domain is mere to inhibit the biological activities of MH2 domains by a direct physical interaction between MH1 and MH2 domains (Kretzschmar et al. 1997). The carboxy-terminal of the receptor-regulated Smads contain a SS(V/M)S motif which is phosporylated by their type I receptors (Abdollah

et al. 1997). This phosphorylation event was shown to be essential for the function of Smads. Upon phosphorylation, the inhibition of MH1 domain is releaved and they form heteromeric complexes with Smad4 to enter into the nucleus (Macias-Silva et al. 1996). Again, it is the MH2 domain of Smad2 and Smad1 which interacts physically with Smad4. In nucleus these complexes are able to interact with other proteins and act as regulators of gene expression (Lagna et al. 1996).

As inhibitors of TGFβ/BMP signalling, Smad6 and Smad7 constitute a distinct class of Smads. They lack the SSXS motif at their carboxy terminal. Smad7 is able to block the activation of target genes, induced by TGFβ, probably by association with TGFb receptor complexes (Hayashi et al. 1997, Nakao et al. 1997). Smad6 has also an inhibitory effect, but whether its function is specific for BMP signalling or it is a general inhibitor has yet to be clarified (Imamura et al. 1997).

Are the Smad proteins targets of other kinases and signalling pathways? The cellular signalling pathways are complex and dynamic processes. They may recruit the same substrates and have synergistic or antagonistic effects. Interestingly, Smad1 is shown to be phosphorylated at serine residues in the linker region by Erk MAP kinases in vitro and in vivo. This phosphorylation inhibits the nuclear accumulation of Smad1. Thus Erk kinase pathway can have an antagonistic effect on BMP induced signalling (Kretzschmar et al. 1997). There is also evidence for a similar mechanism for Smad2 (De Caestecker et al. 1998).

1.2.2.3 Transcriptional activation by Smads

One of the first evidence came from the fact that when fused to GAL4 DNA-binding domain, C-terminals of Smad1, Smad2 and Smad4 are able to act as transcriptional activators (Liu et al. 1996). A study in Xenopus embryos have shown this hypothesis to be true also in vivo. In response to activin or TGFβ, a complex called activin-response factor (ARF) is formed, and binds to the promoter region of Mix2, an immediate-early activin response gene. This complex contains both FAST1, a winged-helix transcription factor and XSmad2. The MH2 domain of XSmad2 interacts with FAST1 (Chen et al 1996). The third component of ARF is identified as Smad4 and both its MH1 and MH2 domains are essential for the stimulation of Mix2 reporter, contributing to DNA-binding and transactivation functions respectively (Chen et al. 1997). A similar example is activation of Xenopus forehead gene directly by C-terminal of XSmad2 via defined regulatory regions (Howell and Hill 1997). The dissimilarity between these regulatory sequences can be best expained that either Smads interact with different transciption factors upon nucleus entry or there is dose-dependent action of activin. While these target genes are restricted to Xenopus, Smads regulate directly the TGFβ inducible human type VII collagene gene expression (Vindevoghel et al. 1998).

A recent study (Moustakas and Kardassis 1998) have shown that in HepG2 hepatoma cell lines which are responsive to TGFβ , Smad proteins are able to regulate and increase the hepatic activity of p21/WAF1 expression. It is suggested that Smad3 and Smad4 probably exert this effect by functionally interacting with transcription factor

Sp1 rather than directly binding to DNA. Another study shows that Smad3 and Smad4 are part of a nuclear complex that directly binds CAGA boxes in the promoter of PAI-1 upon TGFβ induction. But Smad2 neither participates in it nor binds to these elements (Dennler et al. 1998).

1.3 TGFβ Signalling and Cancer

The resistance to TGFβ induced growth inhibition in tumours probably results from disruption of one of the components of TGFβ signalling pathway. As the understanding of this pathway increased, several proteins shown to be mutated in various cancers and they became strong candidates of tumour suppressor genes.

1.3.1 M6P/IGF2 Receptor

M6P/IGF2 Receptor has distinct activities which are important for the regulation of different molecules such as mannose 6 phospate and IGF2 . IGF2 is a growth and survival factor which is degraded by this receptor. Interestingly, the inactive form of TGFβ also binds to IGF2R and is activated by plasmin with the help of the receptor. Thus, inactivation of this receptor will have two effects. First, it will increase the level of IGF2. Second, it will decrease the active TGFβ levels which may help the hepatocytes to escape from negative-growth effect of TGFβ. Frequent loss of heterozygosity and mutations of IGF2R gene have been reported in HCC (De Souza et al. 1995, Yamada et al. 1997).

1.3.2 TGFβ Receptors

Many cancer cell lines are known to acquire TGFβ resistance. Inactivating mutations in TGFβ receptor type II and type I, can be a cause for TGFβ resistance in cancer. Mutations have been identified in TβR-II gene, in two head and neck squamous cell carcinoma (HNSCC) cell lines (Garrigue-Antar et al. 1995), in cutaneous T-Cell lymphoma cell lines (Knaus et al.1996), in colon and gastric cell lines (Vincent et al. 1997) and in colon cancer cells with MSI (Markowitz et al. 1995). These mutations can reflect genetic changes aquired in culture rather than the real situation in primary cancers. Indeed, until now, TβR-II was shown to be mutated only in sporadic colon (Takenoshita et al. 1995) and endometrial (Lu et al. 1996) cancers with microsatellite instability (MSI), or in hereditary non-polyposis colorectal cancer (HNPCC) cases (Lu et al. 1996) and not in cancers of breast, pancreas and liver (Vincent et al. 1996). Thus TβR-II acts as a tumor suppressor mainly in cancers with MSI, although a very recent study indicates that a HNPCC patient without MSI has a germline mutation in TβR-II gene outside the (A)10 tract (Lu et al. 1998).

1.3.3 Smads

1.3.3.1 Allelotyping studies

Even before the identification of Smad4, 18q loss was seen frequently in many tumour types. But many of these studies are quite confusing because of discrepancies on the percantage and the location of LOH in a tumour type. An extensive study on 12

tumour types concentrated on 18q21.1 using polymorphic markers near Smad4 and Smad2. Significant losses (>20%) were observed in HNSCCs, melanomas, osteosarcomas, in breast, lung, hepatocellular, ovarian, pancreatic and prostatic cancers (Schutte et al. 1996).

1.3.3.2 Mutational analysis

Most cell lines, who are insensitive to TGFβ, express intact TGFβ receptors in their cell surface (Vincent et al. 1996). Thus, Smad proteins as mediators of TGFβ signalling are candidate tumor suppressors. Smad4 was first identified as a candidate tumor suppressor gene involved in pancreatic cancer (Hahn et al. 1996). Later on, it was shown to be mutated frequently also in colorectal cancers (Thiagalingam et al. 1996, Takagi et al. 1996) , but infrequently in ovarian cancers (Schutte et al. 1996), in lung cancers (Nagatake et al. 1996), in gastric carcinomas (Powell et al. 1997) and recently in biliary tract carcinoma (Hahn et al. 1998). Smad2 mutations are observed only in colorectal (Riggins et al. 1997, Eppert et al. 1996) and in lung (Uchida et al.1997) cancers (Table 5). Recently germline mutations were found in Smad4 in families with juvenile polyposis syndrom (Howe et al. 1998).

Majority of the mutations are harboured within the carboxy terminal MH2 domain. The crystal structure of C- terminal domain of Smad4 revealed that it forms a trimer in crystal. Most of the missense mutations are in trimer-interface and disrupt the homo-oligomerization. These mutation also prevented formation of heteromeric complexes between Smad4 and Smad2. It can be concluded that homomerization of Smad4 is prerequisite for heteromerization and signal transduction (Shi et al. 1997).

A conserved Arginine residue in the N terminal of is shown to be mutated both in Smad4 and Smad2 . N-terminal of the Smads have an inhibitory effect on Smad function by interacting with its C-terminal. It was shown that these mutations increase the affinity of N-terminal on C-terminal and create an autoinhibitory effect. Such mutations abolish the function of phosphorylation and thus formation of heteromeric complexes (Hata et al. 1997).

Table 5: Somatic mutations in Smad2 gene.

Mutation	**Codon**	**Exon**	**Tumour type**	**Reference**
Deletion	345-358	9	Colorectal	RIGGINS et al.
CTT- CGT	L440R	11	Colorectal	EPPERT et al.
CCT- CAT	P445H	11	"	- " -
GAC-GAG	D450E	11	"	"
CGC-TGC	R133C	4	"	"
GAC-CAC	D450H	11	Lung	UCHIDA et al.
Deletion	434-436	11	"	"

1.3.3.3 Knockout and Transgenic Mouse Models

Knockout mice for Smad4 die before day 7.5 of embryogenesis. The growth retardation, lack of gastrulation and impaired differentiation is similar to the phenotype of Bmp4 mutant embryos. Additionally, the anterior-posterior axis is also disrupted in rescued embryos. These data suggests that a central signalling molecule of TGFβ related pathways, Smad4 is essential for embryogenesis (Sirard et al. 1998).

Similarly Smad2 mutant embryos demonstrated that Smad2 function is essential for the establishment of anterior-posterior polarity, and development of the primitive endoderm lineage. These mutants entirely lack embryonic germ layer tissues (Waldrip et al. 1998). Interestingly, TGFβ1-3 or activin deficient mice are normal at birth (Schull et al. 1992). This fact can be explained either by redundancy of these molecules or that Smad2 acts downstream of other yet unidentified ligands.

Mice which are transgenic for TGF-α under the MMTV promoter develop mammary tumours. In mice transgenic both for TGF-α and TGF-β there is resistance to mammary tumour formation, probably by reduction of epithelial mass (Pierce et al. 1995)

A transgenic mouse model with keratinocyte-targeted expression of TGFβ1 suggests that TGFβ1 has biphasic effects during multistage carcinogenesis. At early tumor promotion stages of carcinogenesis, TGFβ1 acts as a growth inhibitor of keratinocytes and inhibits benign tumour outgrowth. At later stages the cells that were able to escape this inhibition through genetic alterations in TGFβ signalling, have an elevated

level of TGFβ1 which enhances their malignant conversion. The mouse model clearly shows that the TGFβ1 effects the malignant phenotype by altering the expression of extracellular matrix and cell adhesion molecules rather than by evasion of host immune surveillance, enhancement of angiogenesis or elevated apoptosis (Cui et al. 1996).

Compound heterozygote mice , carrying both mutant Smad4 and APC genes were viable and they lost the wild-type allele in tumour tissue. Interestingly, the intestinal tumours progressed into a malignant phenotype at a much earlier stage than the Apc-knockout mice. Thus, Smad4 mutations are sufficient to bring the polyp adenomas induced by APC mutations , in a malignant stage without any other genetic changes. (Takaku et al. 1998).

2. AIM

As discussed before, TGFβ has a strong anti-proliferative effect on normal hepatocytes. Nevertheless, the malignant hepatocytes express TGFβ and continue to proliferate. As observed in other cancers and some hepatoma cell lines escape from anti-proliferative effect of TGFβ might be an important step during hepatocellular carcinogenesis.

Therefore, we think that TGFβ signalling pathway is involved in hepatocellular carcinogenesis. Smad2 is a direct mediator of TGFβ response and was shown to be mutated in several cancers. So we concentrate our efforts on the genetic alterations of Smad2 in liver cancers. Smad2 is a 467 aa protein coded by 11 exons and is located near Smad4 on 18q21.1 where LOH was observed in some cancers (Takenoshita et al 1998).

The aim of this study is to check whether the genetic abnormalities of Smad2 gene are involved in hepatocellular carcinoma.

3. MATERIALS AND METHODS

3.1 Tumour Specimens and cell lines

Genomic DNA of 35 tumours were chosen from a collection as published in (Unsal et al. 1994). The patients were from South Africa(n=11), China (n=8), Mozambique(n=7), Japan (n=6), and Germany (n=3). 80 % percent of the samples are HBV positive and 20 % had p53 mutations. From the informative cases(n=20), 20% had cirrhosis (table 6).

Six hepatoma cell lines were used in this study are HepG2 (Aden et al. 1979), Hep3B (Aden et al. 1979), HuH7 (Nakabayashi et al. 1982), Mahlavu (Alexander et al. 1984), FOCUS (He et al.1984) and PLC/PRF/5 (Alexander et al. 1976).

3.2 Solutions:

10 x TBE Buffer Solution

108 g Tris

55 g Boric acid

8.3 g EDTA

are dissolved in 1 lt of deionized water until its pH is 8.3

Table 6: Characteristics of tumour samples

TUMORS	P53	HBV	Smad2	Stade	Cirrhosis	Metastasis
T8 (SA-L)	WT	+	N	Early	No	No
T12 (SA-T)	WT	-	N	Late	Yes	Yes
T15 (M)	249 Mut	+	N	Late	No	No
T17 (SA-L)	WT	-	N	Late	No	No
T19 (SA-T)	WT	-	N	Late	No	No
T21 (SA-T)	WT (5-7)	+	N	Late	No	Yes
T27 (M)	249 Mut	+	N	Late	Yes	Yes
T29 (M)	249 Mut	-	N	Early	No	No
T31 (SA-T)	WT	+	N	Late	Yes	Yes
T33 (SA-T)	WT	+	N	Late	No	Yes
T35 (SA-T)	WT	+	N	Late	Yes	Yes
T37 (M)	249 Mut	+	Alt. Ex11	Late	Yes	Yes
T39 (SA-S)	WT	+	N	Late	Yes	No
T43 (SA-T)	286-8bp-del	+	N	Late	No	No Infor.
T47 (M)	157 Mut	+	N	Late	No	No
T49 (SA-C)	WT	+	Alt. Ex10	Late	No	No
T51 (M)	WT	+	N	Late	No	Yes
T53 (M)	WT	+	N	Late	Yes	No
T73 (G)	WT (Ex 7-9)	+	N	Late	Yes	Yes
T80 (G)	WT	+	N	No Infor.	Yes	No Infor.
C2 (C)	WT	+	N	No Infor.	No Infor.	No Infor.
C3 (C)	WT	+	N	No Infor.	No Infor.	No Infor.
C6 (C)	WT	+	N	No Infor.	No Infor.	No Infor.
C9 (C)	WT	+	N	No Infor.	No Infor.	No Infor.
C10 (C)	249 Mut	+	N	No Infor.	No Infor.	No Infor.
C12 (C)	WT	+	N	No Infor.	No Infor.	No Infor.
C15 (C)	WT	+	N	No Infor.	No Infor.	No Infor.
C23 (C)	WT	+	N	No Infor.	No Infor.	No Infor.
J1T (J)	WT	+	N	No Infor.	No Infor.	No Infor.
J3T (J)	WT (Ex 5-7)	+	N	No Infor.	No Infor.	No Infor.
J4T (J)	WT (Ex 7-9)	+	N	No Infor.	No Infor.	No Infor.
J8T (J)	WT (Ex 7-9)	-	N	No Infor.	No Infor.	No Infor.
J9T (J)	WT (Ex 7)	-	N	No Infor.	No Infor.	No Infor.
J10T (J)	WT (Ex 7)	-	N	No Infor.	No Infor.	No Infor.
G5 (G)	WT (Ex 5-7)	+	N	No Infor.	No Infor.	No Infor.

The samples were from South Africa-Lesotho (SA-L), South Africa-Transkei (SA-T), South Africa-Swaziland (SA-S), South Africa-Caucasian population (SA-C), from Mozambique (M), Germany (G), Japan (J) and China (C).

SSCP Loading Buffer Solution

95 % Formamide

0.04 % Bromphenol blue

0.04 % Xylene Cyanol

10 mM NaOH

6 x Loading Buffer Solution

30 % Glycerol

0.04 % Bromphenolblue

0.04 % Xylene Cyanol

Δ dH_20

Agarose Gel Solution for PCR

30 ml 1 x TBE

0.6 g Agarose

heated in microwave for 1 min. and added 1 ml of EtBr

Polyacrylamide Gel Solution for SSCP

the stock solution is 50 %

49.5 g Acrylamide

0.5 g Bisacrylamide

Add ddH_20 to 100 ml

3.3 Expression of cell lines

3.3.1 PCR

Primers: The forward (F) and reverse (R) primers for the amplification of Smad2 coding region are as follows (Riggins et al.) :

TC 104 (F): 5` GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGG TAA GAA CAT GTC GTC CAT C 3'

TC 103 (R): 5' TTT CCA TGG GAC TTG ATT GG 3'

TC 104 (F) includes signals for transcription and translation

Polymerase Chain Reaction (PCR) conditions:

Reaction mixture: for each exon the PCR reaction was performed in 25 ml of mixture containing:

1 x Buffer

1.5 mM $MgCl_2$

0.8 mM dNTP mix

20 pmol F primer

20 pmol R primer

50 ng DNA

1 u Taq polymerase

Δ H_2O

PCR conditions for amplification consisted of a 3-min denaturation step at 95°C, followed by 35 cycles of 40 sec at 95°C, 40 sec at 56° and 90 sec at 72°C. After 35 cycles a last step at 72°C for 7 min was added.

3.3.2 Agarose gel electrophoresis

5 ml of PCR product was mixed with 1 ml of 6 x loading buffer and loaded into 1.5 % agarose gel electrophoresis. They were run 80V for 40 min and then visualised under UV light.

3.4 Single-Strand Conformation Polymorphism (SSCP) Analysis

3.4.1 PCR conditions:

Primers: Intronic or partly intronic primers were designed by myself. The exact positions of the primers can be found in Appendix A. The forward(F) and reverse (R) primers for the amplification of each exon are as follows:

exon 4 : F: 5' TCTGATAGTGGTAAGGGTTT 3'

R: 5' GTCTCTTGATGGTCGTCTC 3'

exon 8 : F: 5' CAGTTACTTACTCAGAACCT 3'

R: 5' GCCTACATTATGAGTATACAG 3'

exon 9: F: 5' CCAAAGTCACACTGAAATAG 3

R: 5' AGCAAGTTGACATGATAGG 3'

exon 10: F: 5' GCATGCTCATATTCTAAAAC 3'

R: 5' ACTGTGGAAATTTAAGAACC 3'

exon 11: F: 5' GCCTGTGGACTTGAATTTCAT 3'

R: 5' GGACTTGATTGGTGAAGCTTT 3'

C-term : F: 5' CAGGGTTTTGAAGCCGTCTA 3'

R: 5' CTTGAGCAACGCACTGAAGG 3'

Polymerase Chain Reaction (PCR) conditions:

Reaction mixture: for each exon the PCR reaction was performed in 25 ml of mixture containing:

1 x PCR Buffer

1.5 mM MgCl2

0.04 mM dNTP mix

[33P] 0.5 μCi dCTP

20 pmol F primer

20 pmol R primer

50 ng DNA

1 u Taq polymerase

Δ H_2O

Reaction conditions: the reaction condition is the same for each exon except the anneling temparature (Ta). The Ta for each exon is as follows:

exon 4: 56°C	exon 8: 53°C	exon 9: 57°C
exon 10: 50°C	exon 11: 56°C	C-term: 60°C

PCR conditions for amplification consisted of a 3-min denaturation step at 95°C, followed by 30 cycles of 30 sec at 95°C, 30 sec at Ta and 30 sec at 72°C. After 30 cycles an additional step at 72°C for 5 min was performed.

3.4.2 Polyacrylamide gel electrophoresis:

The mixture contains:

1 x TBE

10 % Glycerol

20 % polyacrylamide gel solution

add ddH_2O to 100 ml

After mixed precisely and degased, 400 ml of 10% Ammonium persulfate (APS) and 40 ml of Temed is added for a mixture volume of 100 ml. This solution is poured into gel apparatus and left for polymerization for 2 hours.

SSCP reaction:

Mixture:

2 ml of PCR product was mixed with 8ml of SSCP loading buffer in an Eppendorf ® tube.

Denaturation:

The tubes were denatured at 95°C for 2 min and then put immediatly into ice.

Loading:

The samples were loaded into an electrophoresis apparatus and electrophoresed at 4°C , at constant 45 W for 14 hours.

3.4.3 Exposure and development

Then the gel was dried on a 3M Wattman® paper and exposed to X-ray film for 3 days. The exposed film was developed in developer machine.

3.4.4 Automated sequencing

The sequencing reactions were performed and analysed in ABI Prism 377 Sequencer, using the same primer as used for SSCP analysis.

4. RESULTS

4.1 Smad2 expression in hepatoma cell lines

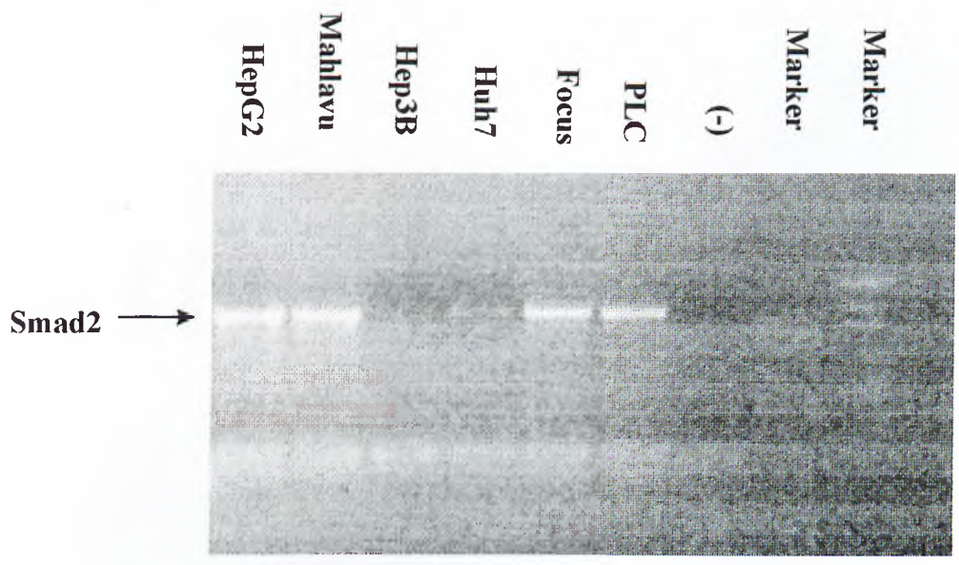

Figure 1: Expression of hepatoma cell lines

Smad2 expression was not exactly known in the hepatoma cell lines. The whole coding region of six hepatoma cell lines are amplified by PCR (conditions described above). Single band was observed with expected size for all cell lines tested. All the six cell lines express Smad2. There are no large deletions in any of the cell lines. Although four of them had similar amounts of amplified products, a nested PCR from these products still shows that Hep3B and Huh7 are less amplifyible (Figure 1).

4.2 SSCP analysis of hepatoma cell lines

Figure 2: SSCP analysis of Smad2(C-term) for hepatoma cell lines in 10% Acrylamide gel

Figure 3: SSCP analysis of wildtype and mutant p53

SSCP analysis is performed for a part of the conserved C-terminal domain of Smad2 in five hepatoma cell lines. The upper and lower bands correspond to each single-stranded DNA of the amplified region. No different migration pattern (e.g. no shifted

bands) were observed (Figure 2). SSCP analysis for exon 7 of wildtype and mutant (p53248 and p53249) p53 genes are used as a control for the efficiency of SSCP conditions. Both mutants had a distinct migration pattern from the wild-type exon of the gene.

4.3 Sequencing analysis of hepatoma cell lines.

As to confirm the SSCP results, the PCR products of Smad2 (C-term) was sequenced. No alteration was detectable by sequencing data, confirming the SSCP results in Figure 1.

4.4 SSCP analysis of tumour samples.

Exons 4,8,9,10 and 11 of Smad2 gene were analysed by SSCP analysis in 35 samples. The results gave us three distinct bands, one far below of the gel , and the other two in upper side of the gel relatively. The intensity of the lowest band was higher, and due to its migration pattern and intensity this band corresponds to the double-stranded product. The two upper ones migrate according to their confirmation and they correspond to the single strands (Figure 4)

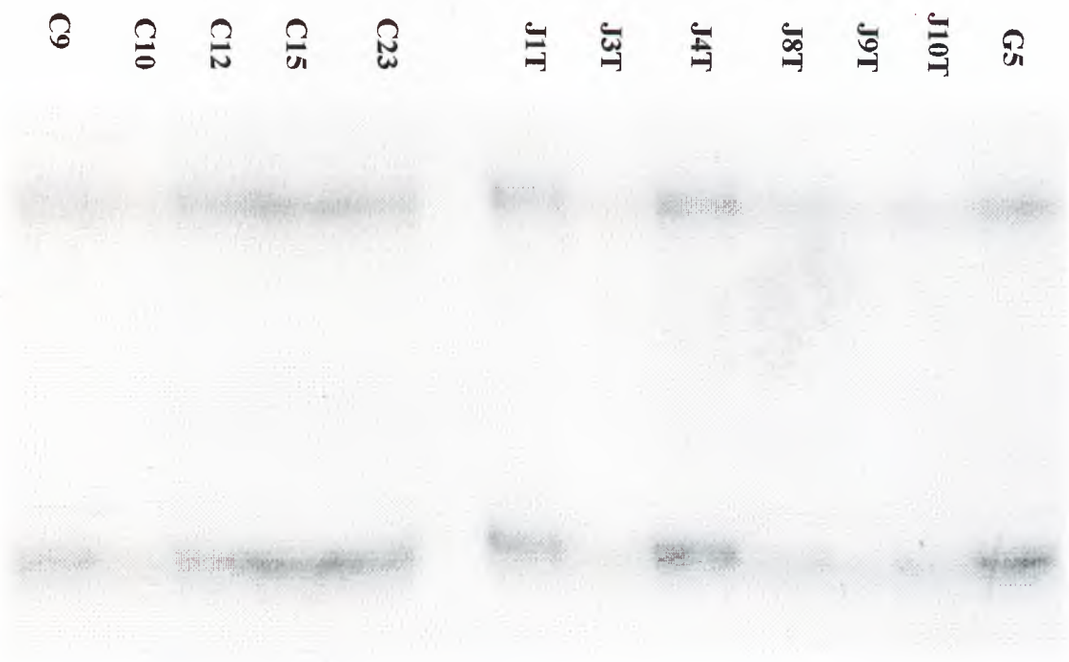

Figure 4: SSCP results of normal migrating bands (exon9)

No alterated bands were observed for any sample of exon 4 , exon 8 and exon 9.

However an alteration was found both for exon 10 and exon 11.

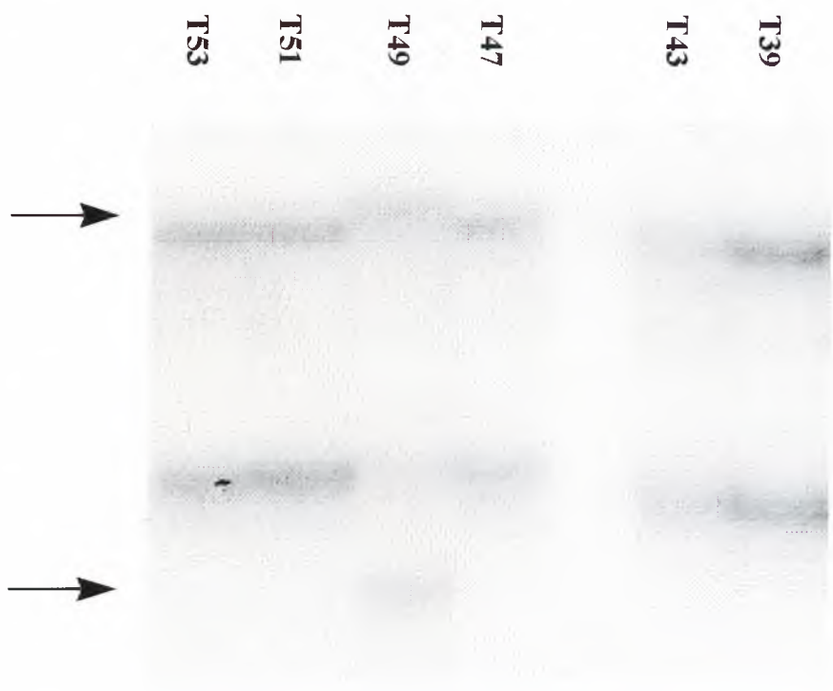

Figure 5: Alteration in T49

Tumour sample T49 showed a different migration pattern from the other tumour samples. Its both single strands migrated differentially from the others. (Figure 5). The band corresponding to the double-stranded product had a lower migration than the other double-strands.

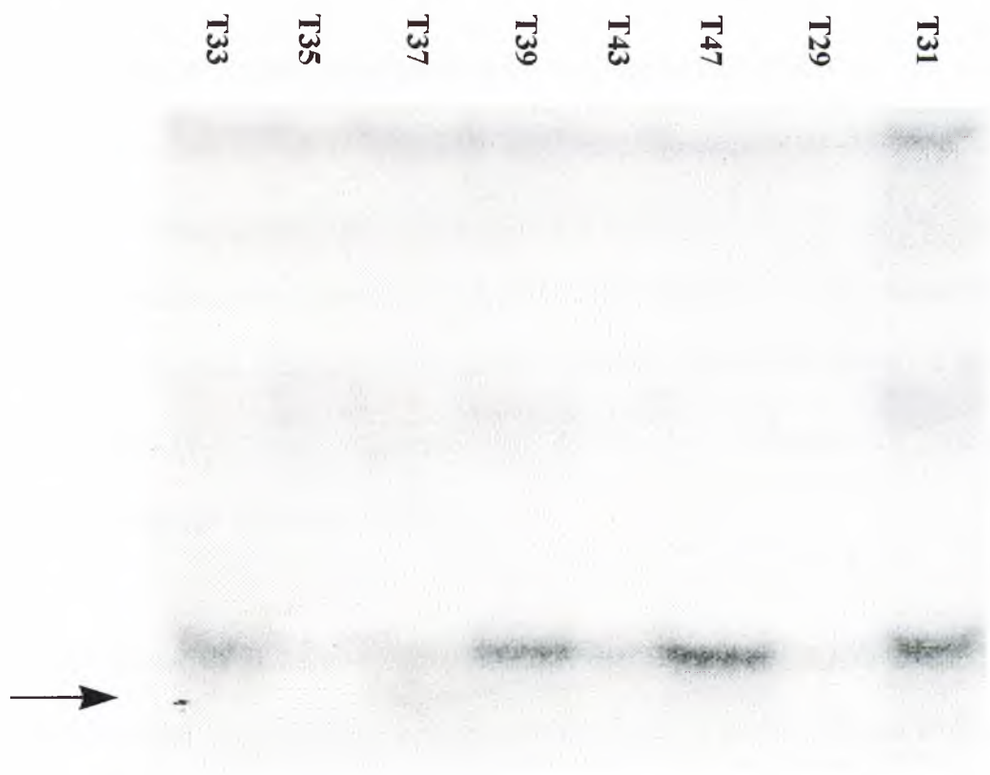

Figure 6: Alteration in T37

Tumour sample T37 showed also a different migration in its exon 11.

The less intense bands between two single strands (the most upper and lower bands) are either artifacts or phantom bands which are observed in many SSCP studies. They appear in each sample tested. Even the more intense bands like T39 or T47 had not such a shifted band below the lower single-strand (Figure 5).

5. DISCUSSION

Members of TGF-β superfamily have very important effects on cell proliferation, differentiation, organ development and morphogenesis. TGF-β1 itself is involved during embryogenesis and differentiation. It is known to be a potent inhibitor of most of the normal cells, mostly of epithelial cells. However, during tumourigenesis the malignant cells continue to proliferate although the TGFβ levels in these tissues increase. It is even suggested that TGFβ1 is a enhancer of malignancy in carcinogenesis (Cui et al. 1996).

This paradox can be best explained that the cancer cells acquire mutations which enable them to escape from negative growth effect of TGFβ. The last three years gave us many evidences that this might be true for many types of cancer. With the identification of TGFβ receptors, especially one of them, TβR-II was shown to be mutated or inactivated. But, unfortunately these mutation were only observed in cancers with microsatellite instability, such as HNPCC and gastric cancers where a (A)10 TβR-II is the target (Vincent et al. 1997). We can even suggest logically; as they are the only known receptors for TGFβ, the effect of TGFβ as an enhancer of malignancy can be accomplished if they remain intact. Smad2, 3 and 4 proteins are shown to be the sole mediators of TGFβ until today. Together with the other Smads they have highly conserved amino- and carboxy-terminal domains. The C-terminal domain was shown to mediate the activity of TGFβ both in invertebrates and in vertebrates, including mammalians (Kretzschmar and Massague 1998). Smad4

became the focus of cancer research when it was shown highly mutated in pancreatic cancers. It is located near to in 18q21.1 a region which shows a high rate of LOH in some cancer types (Schutte et al. 1996). DCC gene which was thought to be the main target of inactivation in this region, is not considered as a tumour suppressor anymore. Interestingly, Smad2 ,the main partner of DPC4 in TGFβ pathway, is also located very near to DPC4. They are inactivated in some cancer types (Riggins et al. 1996, Eppert et al. 1996, Uchida et al. 1997). It is clear that such an inactivation will abolish the effects of TGFβ. It is demonstrated that they are essential for the signalling.

It was long known that both during fibrosis, cirrhosis and during hepatocellular carcinoma there is an increase in TGFβ levels, although the source of the production is still an unsolved question (Bedossa et al. 1995). Although there is one report showing microsatellite instability in hepatocellular carcinoma, the target gene mutations like TβR-II have not been studied (Salvucci et al. 1996). Furthermore, it was shown that they are intact and expressed (Vincent et al. 1996). Some has suggested that the receptors can be downregulated during tumourigenesis, but there is not enough evidence for it (Sue et al. 1995). Therefore, TGFβ related Smads are potential targets of inactivation as the hepatocytes escape from inhibitory effect of TGFβ.

The expression of Smad2 in liver cell lines were not clear and was not studied previously. The amplification of Smad2 cDNA in all six hepatoma cell lines clearly reveales that Smad2 is expressed in them, including Mahlavu cell line which is

unresponsive to TGFβ1 (Ito et al. 1990). We have observed lesser amount of amplification for some cell lines. Studies have to be done in order to clarify Smad2 protein expression in these cell lines. In addition, the SSCP analysis of the most conserved C-terminal region (codons 400-464) from cDNA have demonstrated that there is no alteration in this region of these cell lines. Automatic sequencing data of this region of five cell lines, including Mahlavu confirmed this information.

To detect the significance of Smad2 mutations in liver cancer, we performed SSCP analysis for five exons of Smad2 gene. The four exons cover all the conserved carboxy-terminal region (MH2 domain). This region alone is shown to be sufficient to mediate the TGFβ signalling. Furthermore, all the mutations that are found until today, except one, are located in this domain. In structural analysis, these mutations are shown to impair the homomerization and heteromerization which are essential for transcriptional activity. Other developmental mutations of invertebrates are also located here. The other mutation is found in exon 4, in conserved N-terminal which is thought to have a negative-regulatory effect on C-terminus. Therefore, we chose this exon as the fifth exon for SSCP analysis.

SSCP analysis is more frequently used than the other mutation detection methods like hetero-duplex analysis or DGGE. It is an established technique and shown to detect about 80% of the actual mutations. All the mutational analysis of Smad genes on gene level are carried out by this technique. Furthermore, the shifted single strands give us an advantage to isolate them from the gel, and sequence them after amplification which is difficult for the heteroduplex technique because the heteroduplexes contain normal and mutated strands. Additionally, Smad mutations are

mainly missense mutations for which SSCP is shown to be powerful. Smad2 exons are small exons ideal for SSCP analysis which has the highest resolution between 100-250 base pairs.

SSCP analysis of two patients have shown to have alterations. Interestingly these belong to the last two exons of Smad2 in which 90 % of the mutations are located. One sample was from Mozambique (T37) and the other one (T49) from South Africa. It looks like that no correlation exists between their p53 status because one patient has p53 249 mutation whereas the other one has an intact p53. Both were infected by HBV. We used intronic primers to amplify genomic DNA of the patients. Therefore there is a chance that these alterations are mere polymorphisms in the introns. There is also a probability that these mutations are polymorphisms in the coding regions which seem highly improbable because according to the data published until now, there is no polymorphism in this region. One of the alteration had a lower shift in its doublestrand band which raises the question whether it can be a small deletion. For the other alteration (T37), when the sample which derived from non-tumour tissue was analysed by SSCP , it had a similar abnormal migration pattern like T37. So we may conclude that it is either a polymorphism or a germline mutation.

This is the first study showing a Smad2 alteration in liver cancer. It can be speculated that even though mutations of Smad2 and/or Smad4 (MS thesis of Burcu Irmak) are not frequent, this can be considered first study showing mutations of TGFβ pathway in HCC.

As future perspectives, the mRNA level of Smad2 in different hepatoma cell lines should be confirmed by using the expression level of a house-keeping gene as an internal control. Naturally, the protein expression levels of Smad2 also should be checked in cell lines , to have a final conclusion. The molecular cause for the altered bands have to be determined by sequencing analysis to find out the exact nature of the shift.

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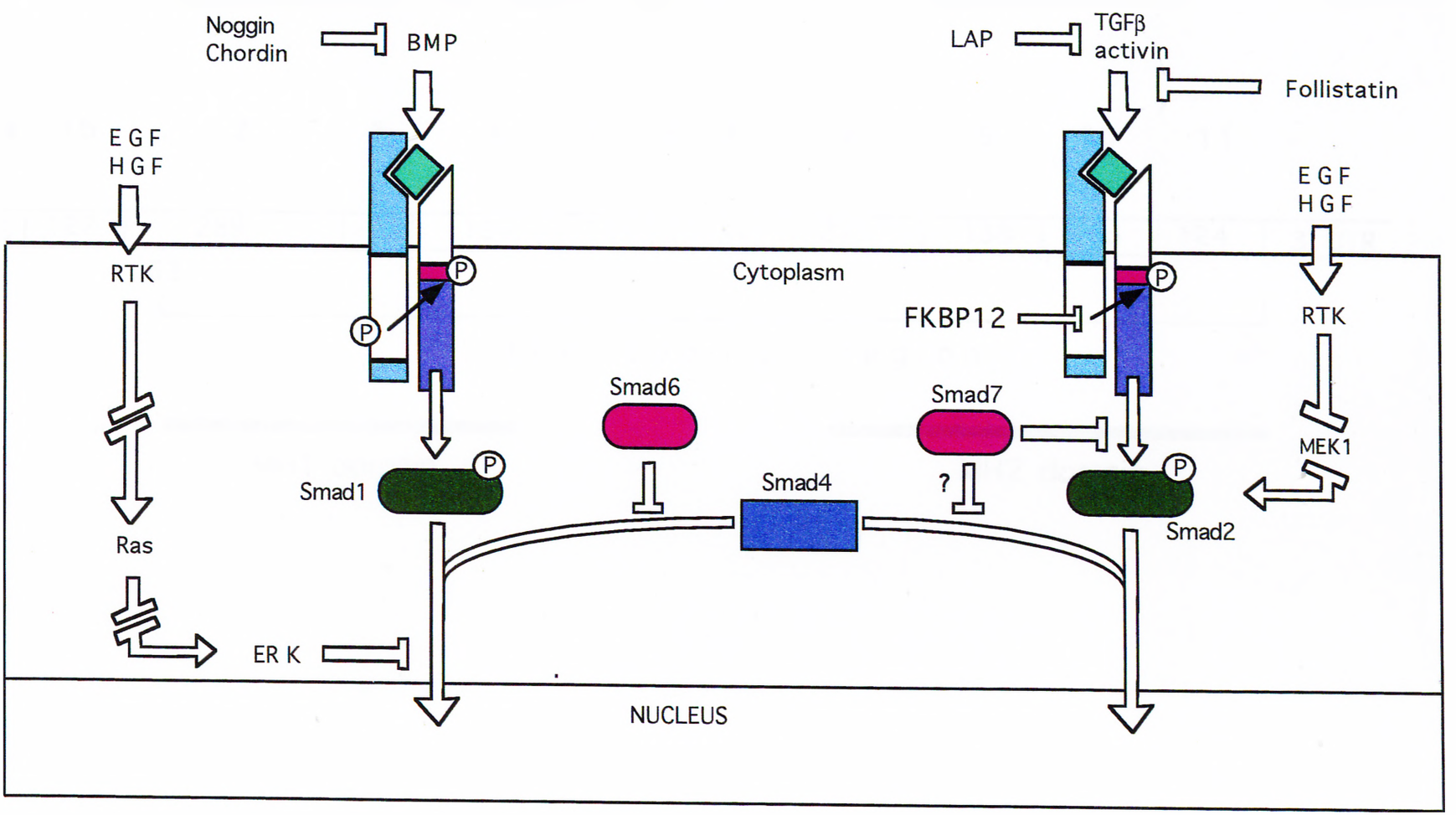
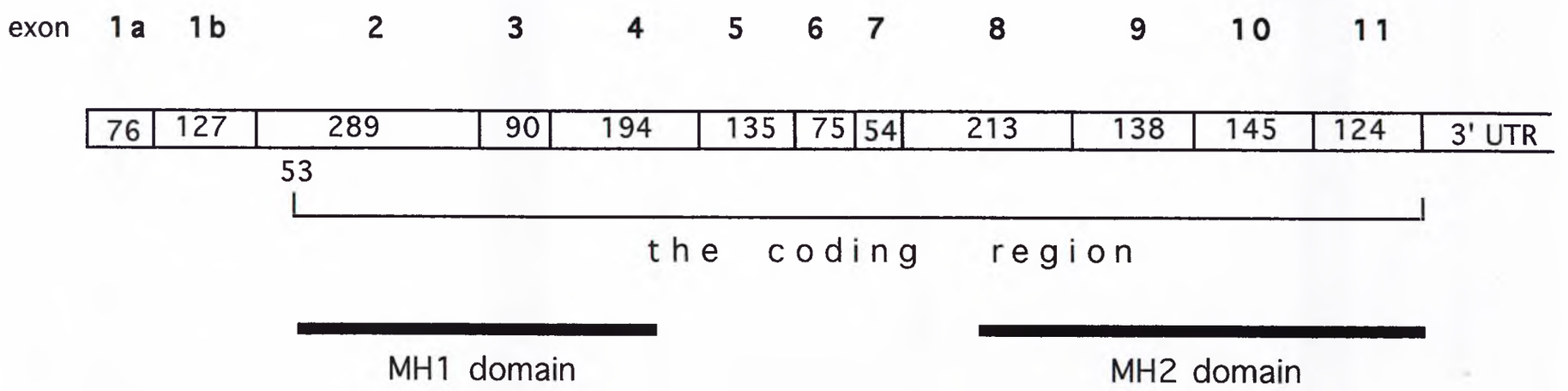
TGFβ signalling pathway



GENETIC STRUCTURE OF SMAD2



SMAD2 gene C-term cDNA

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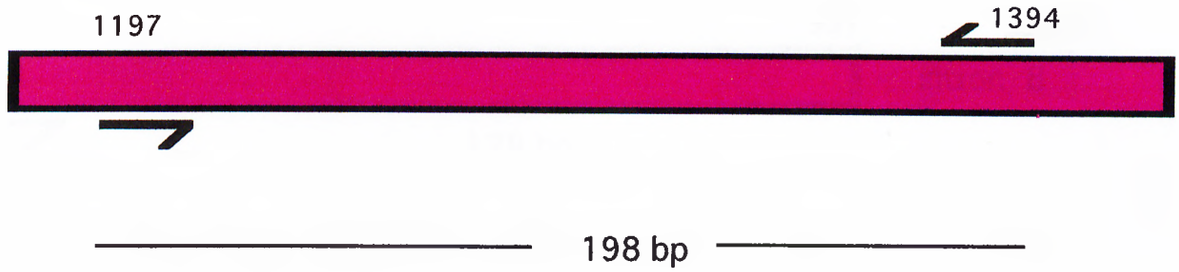
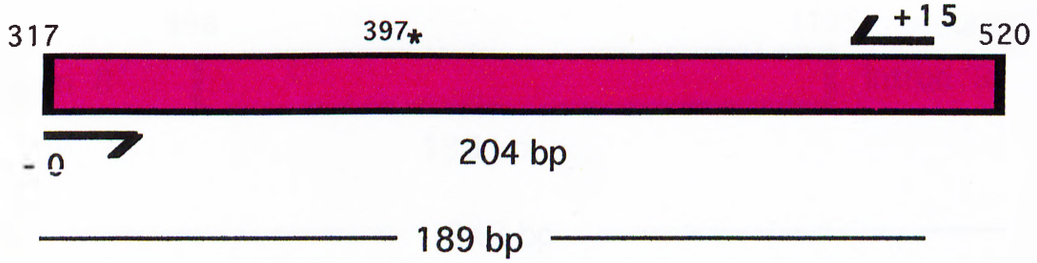
Accession: AF027964



SMAD2 gene EXON 4

Exon size: 204 bp
PCR size: 189 bp
Primers : GA 325-326
Accession: U78728



* Mutation at codon 133

SMAD2 gene EXON 8

Exon size: 213 bp
PCR size: 269 bp
Primers : GA 437-438
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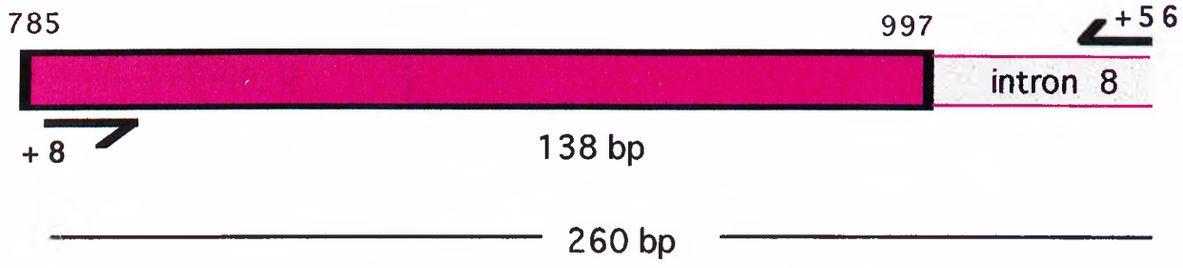

SMAD2 gene EXON 9

Exon size: 138 bp
PCR size: 242 bp
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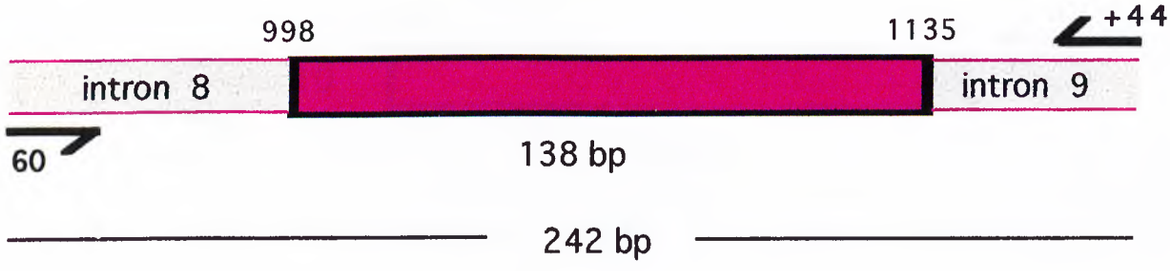

SMAD2 gene EXON 10

Exon size: 145 bp
PCR size: 275 bp
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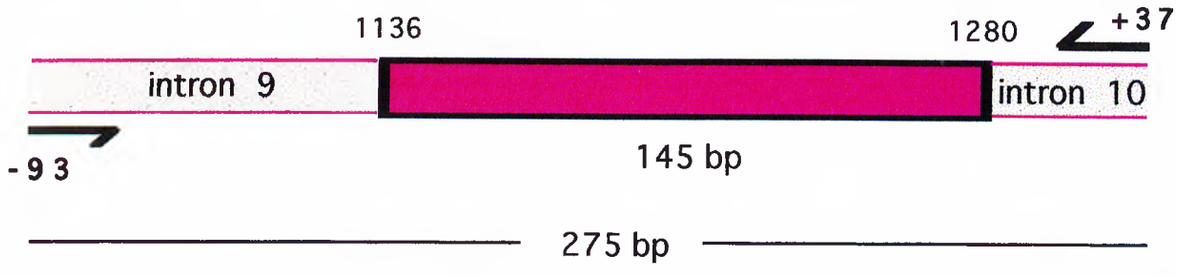
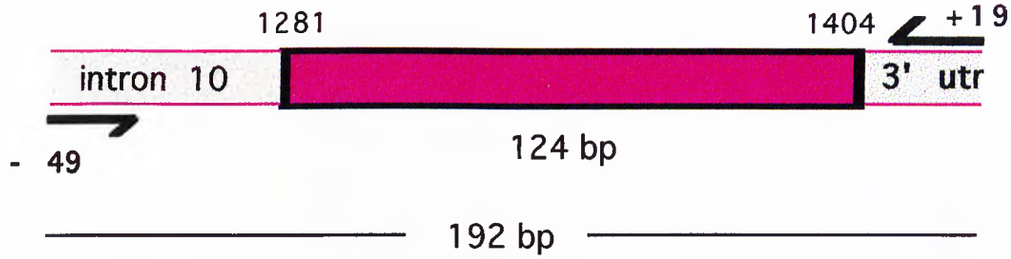
Accession: U78732



SMAD2 gene EXON 11

Exon size: 124 bp
PCR size: 192 bp
Primers : GA 368-369
Accession: U78733



ABI PRISM

Model 377
Version 2.1.1

20•hcp62 147

hcp62 147
Lane 20

Signal G:349 A:670 T:487 C:401
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SN 569
Points 885 to 2400 Base 1: 885

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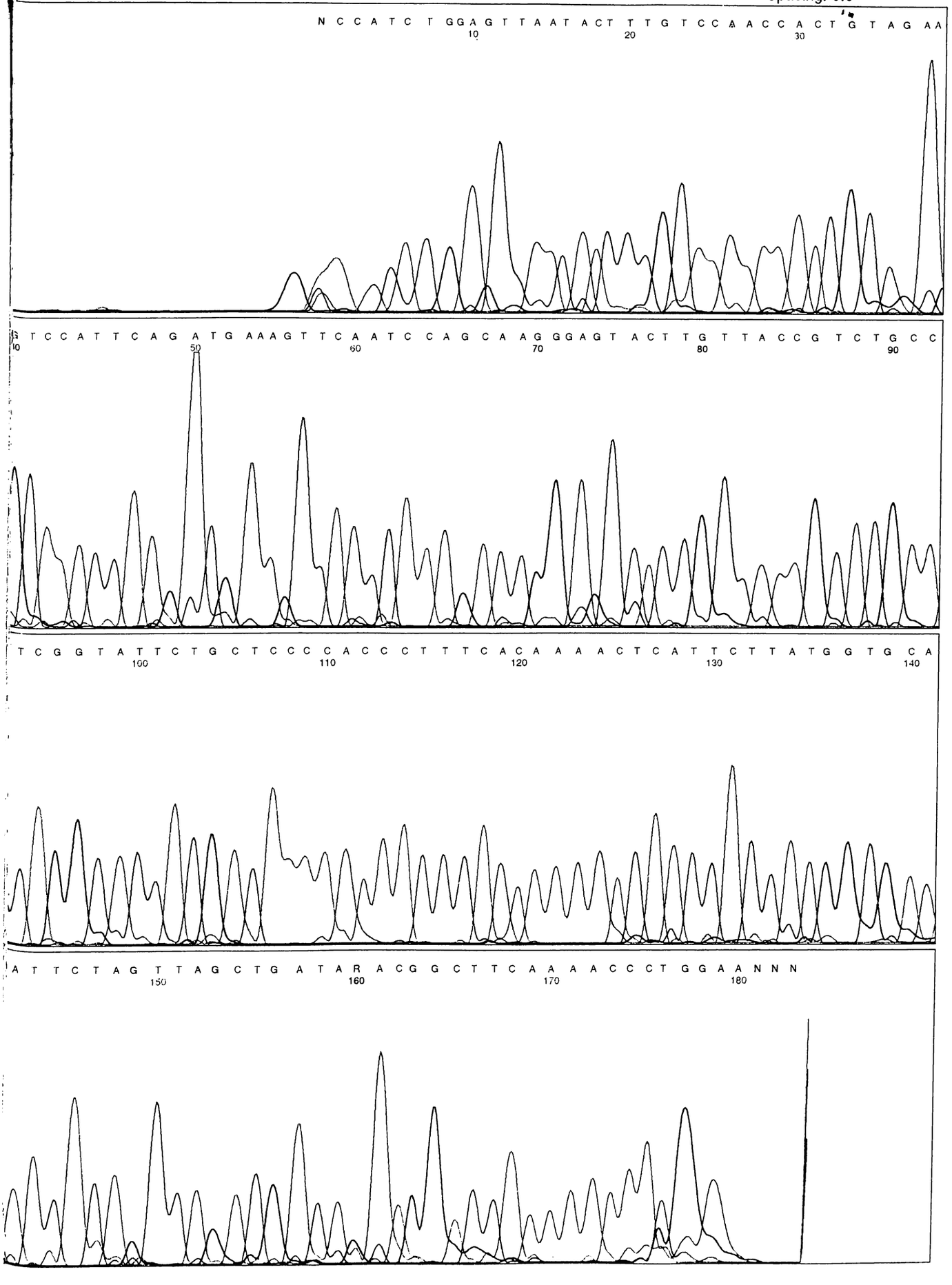
Thu, Oct 09, 1997 18:50
Spacing: 0.0



ABI PRISM

Model 377
Version 2.1.1

21•hcp62 148

hcp62 148
Lane 21

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Points 784 to 2395 Base 1: 784

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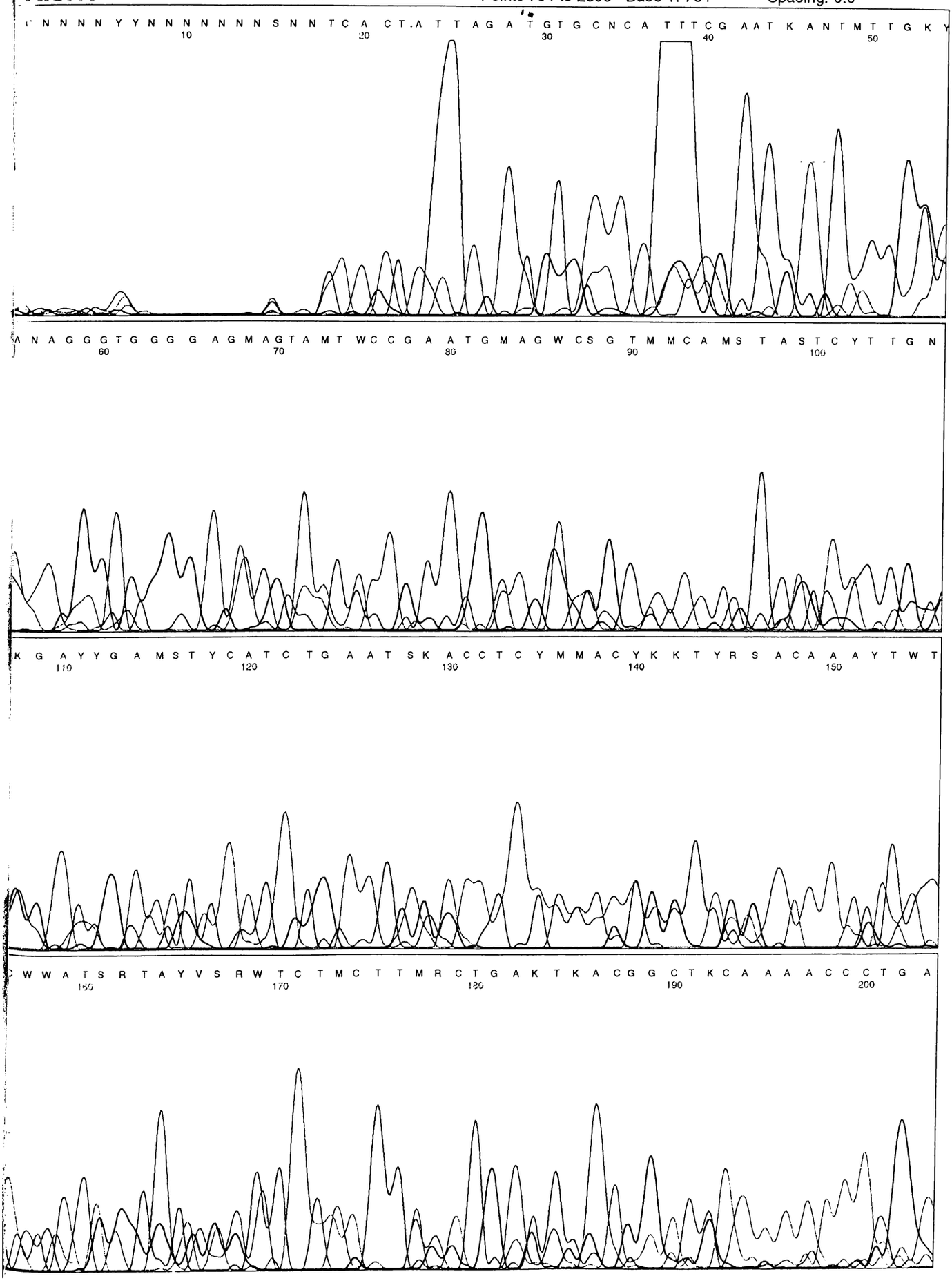

ABI PRISM

Model 377
Version 2.1.1

16•3B 147

3B 147
Lane 16

Signal G:79 A:100 T:123 C:60
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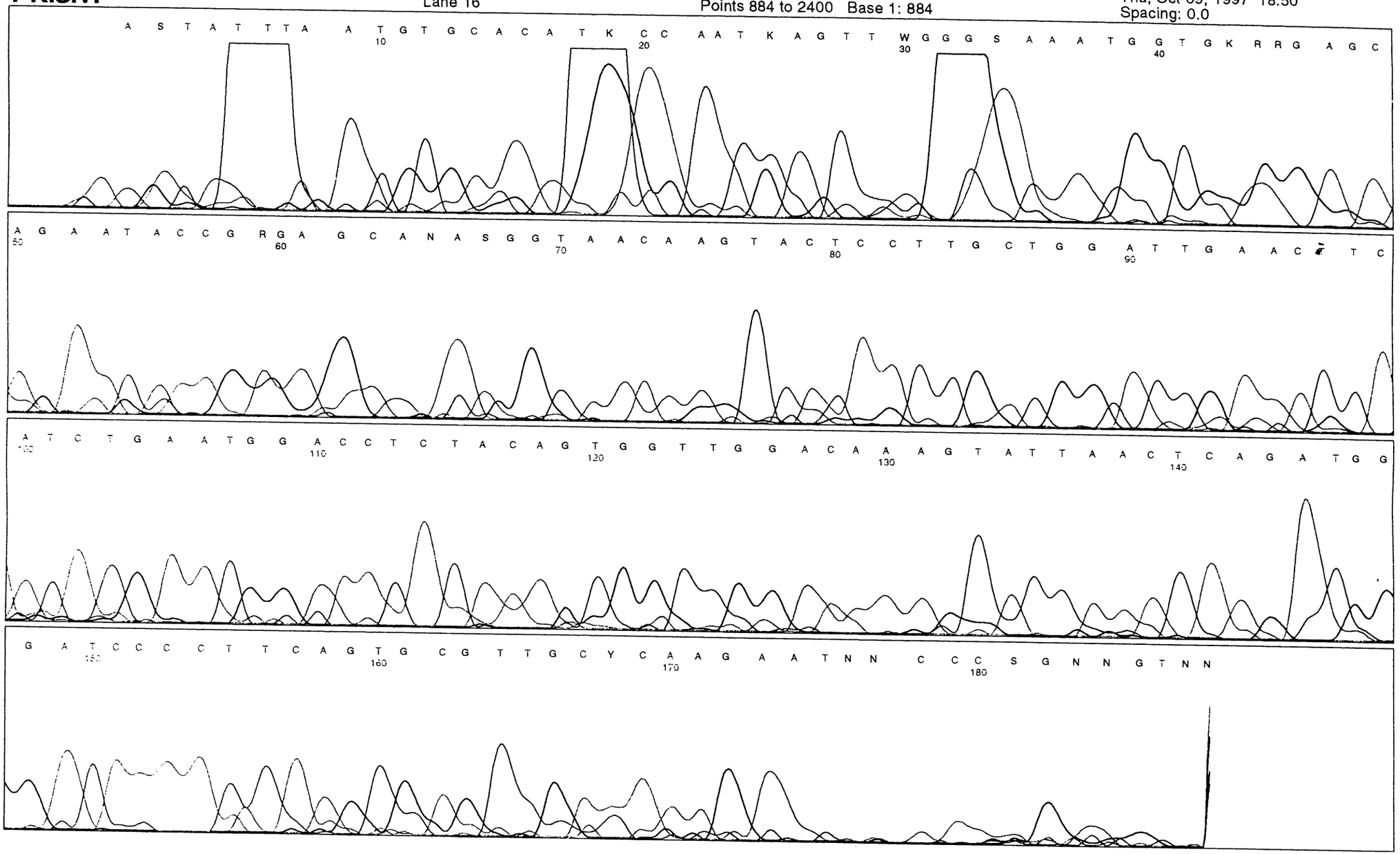

ABI PRISM

Model 377
Version 2.1.1

17•3B 148

3B 148
Lane 17

Signal G:842 A:1394 T:1039 C:701
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Points 840 to 2368 Base 1: 840

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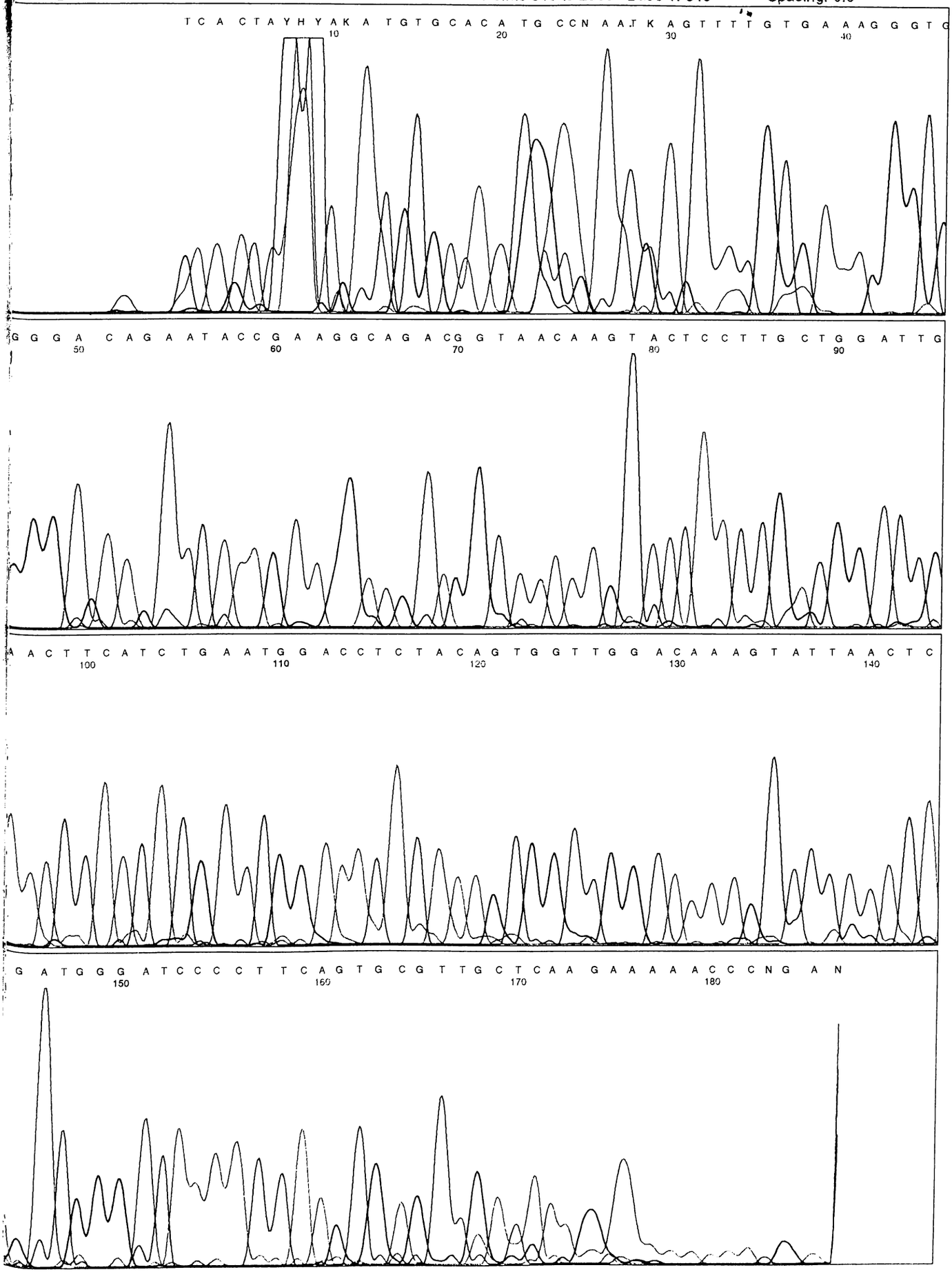

ABI PRISM

Model 377
Version 2.1.1

18•huh7 147

huh7 147
Lane 18

Signal G:667 A:1117 T:804 C:784
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Points 885 to 2388 Base 1: 885

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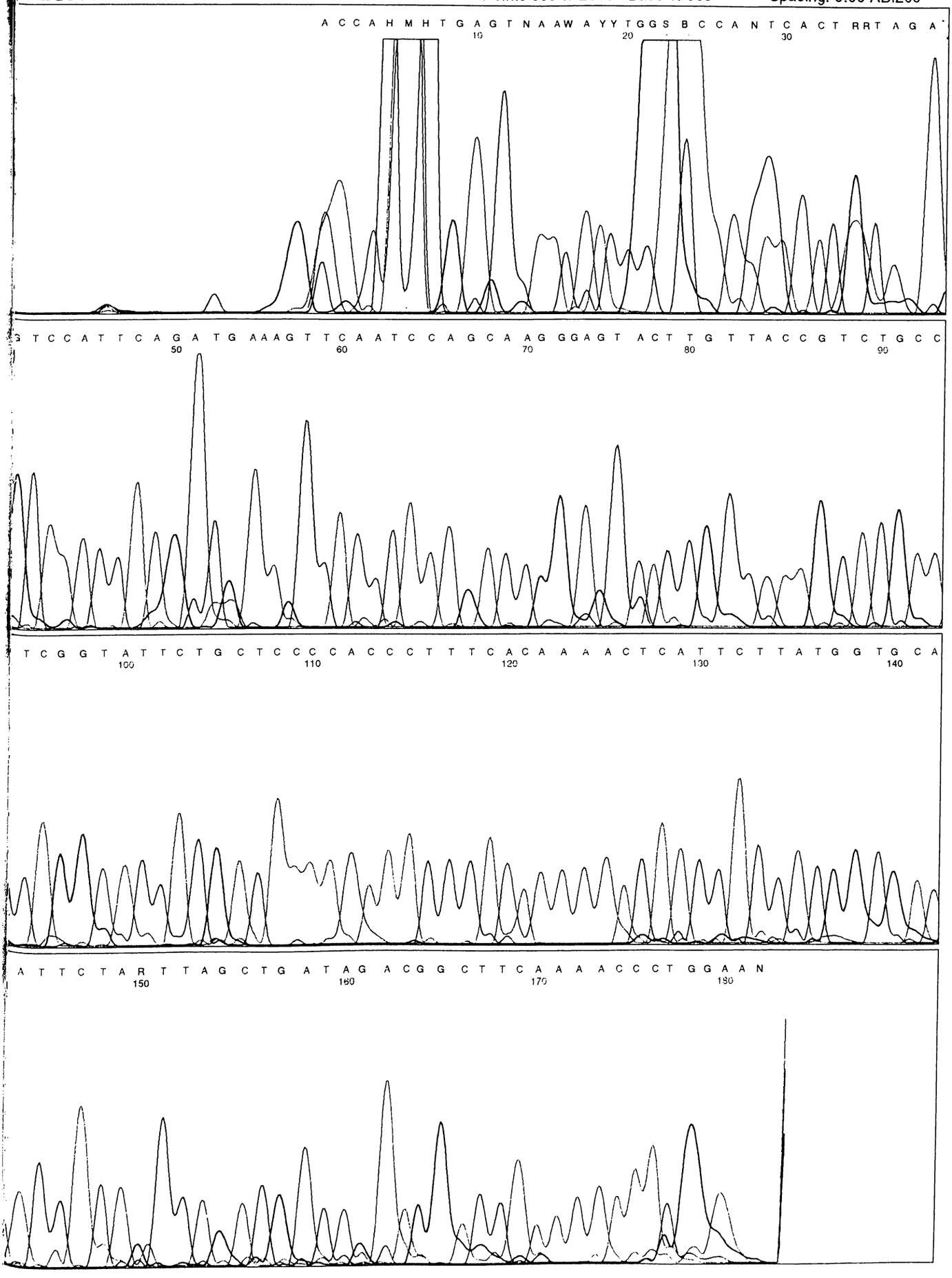

ABI PRISM™

Model 377
Version 2.1.1

19•huh7 148

huh7 148
Lane 19

Signal G:384 A:672 T:455 C:321
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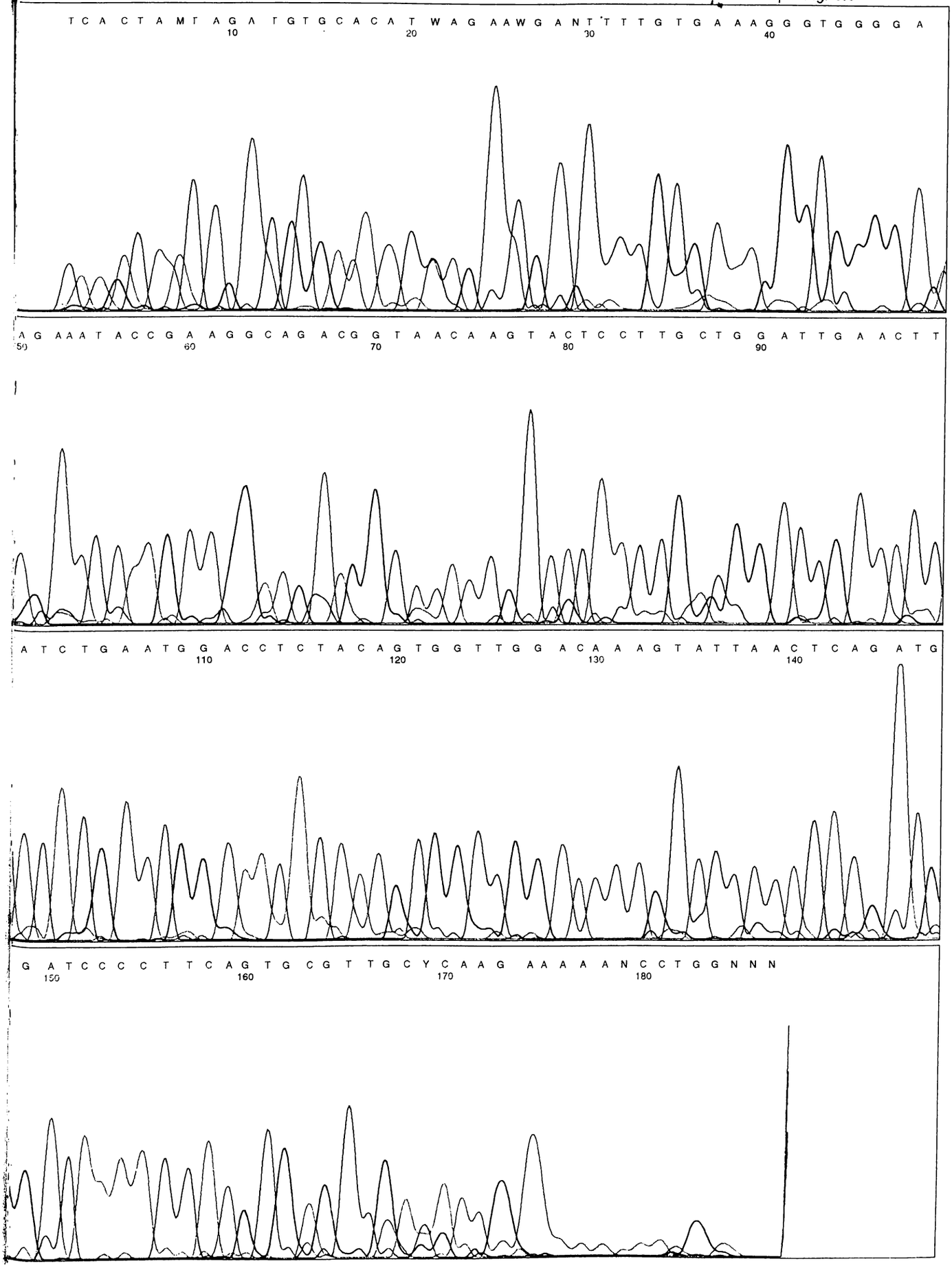

ABI PRISM

Model 377
Version 2.1.1

22•fsm2 147

fsm2 147
Lane 22

Signal G:386 A:670 T:654 C:435
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Points 884 to 2390 Base 1: 884

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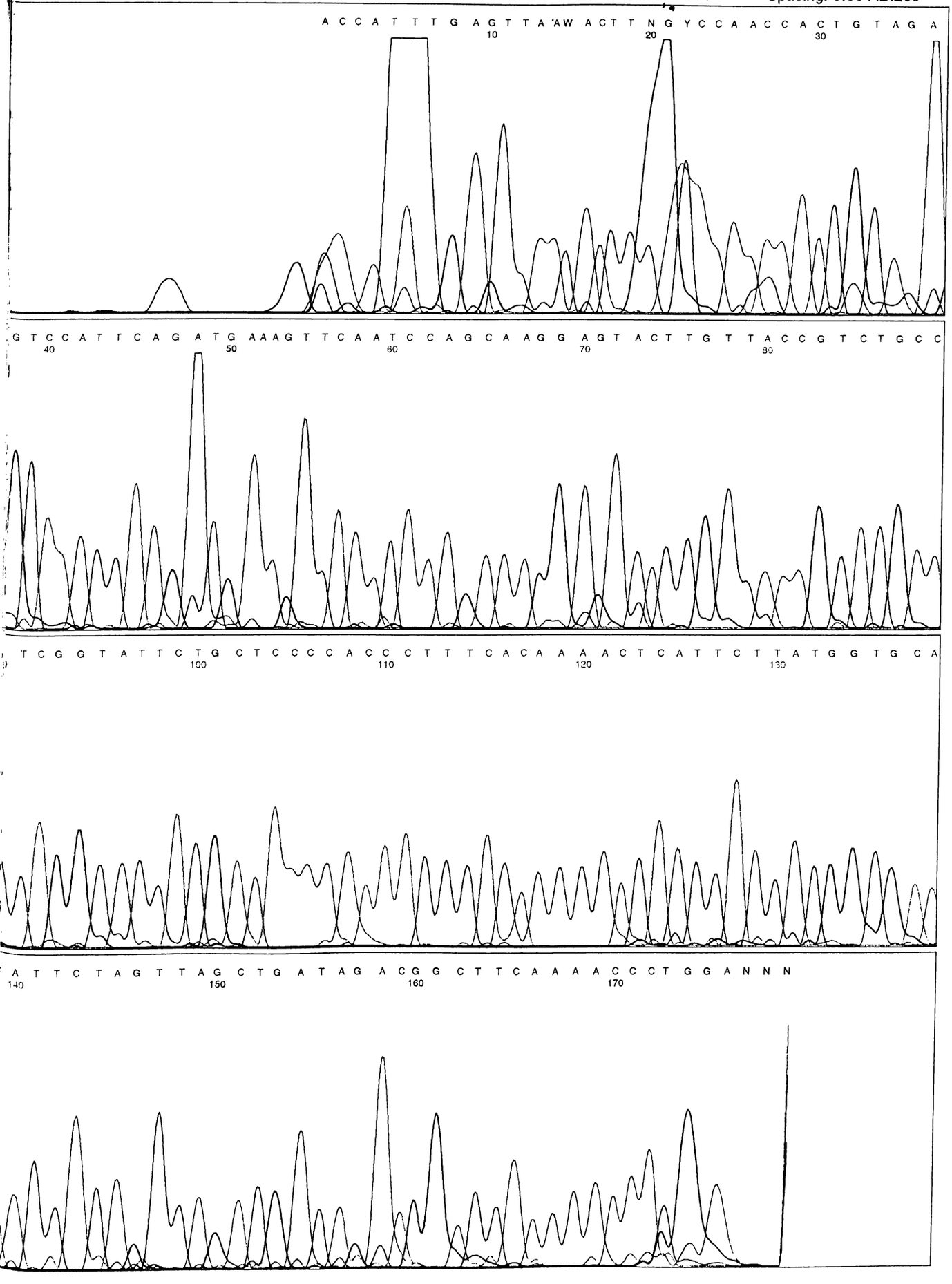

ABI PRISM

Model 377
Version 2.1.1

23•fsm2 148.1

fsm2 148
Lane 23

Signal G:312 A:416 T:339 C:224
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Points 678 to 8268 Base 1: 678

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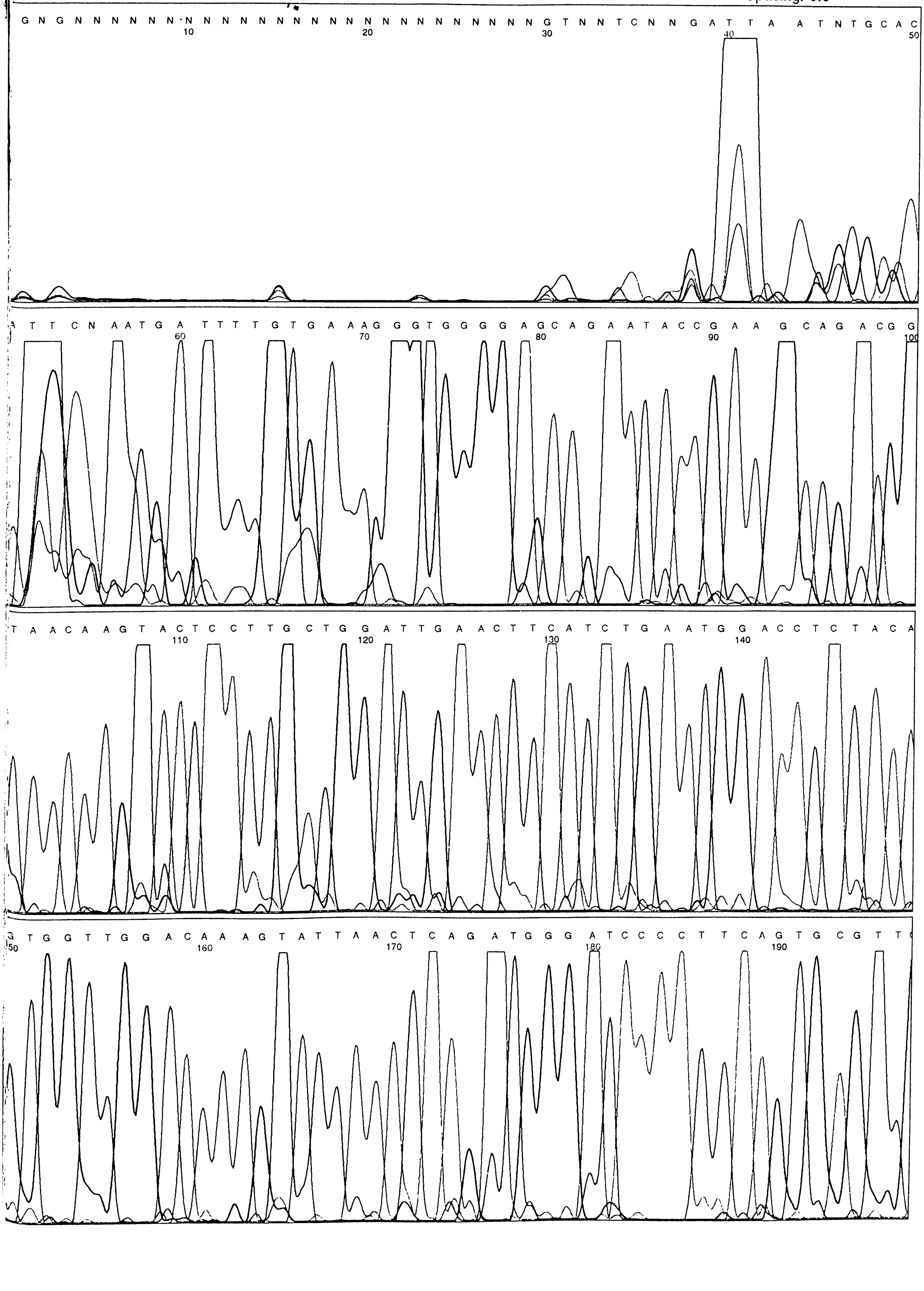

ABI PRISM

Model 377
Version 2.1.1

19•Mahlavu/TC147

Mahlavu/TC147
Lane 19

Signal G:183 A:236 T:168 C:146
DT4%Ac{A Set-AnyPrimer}
SN 569
Points 793 to 2198 Base 1: 793

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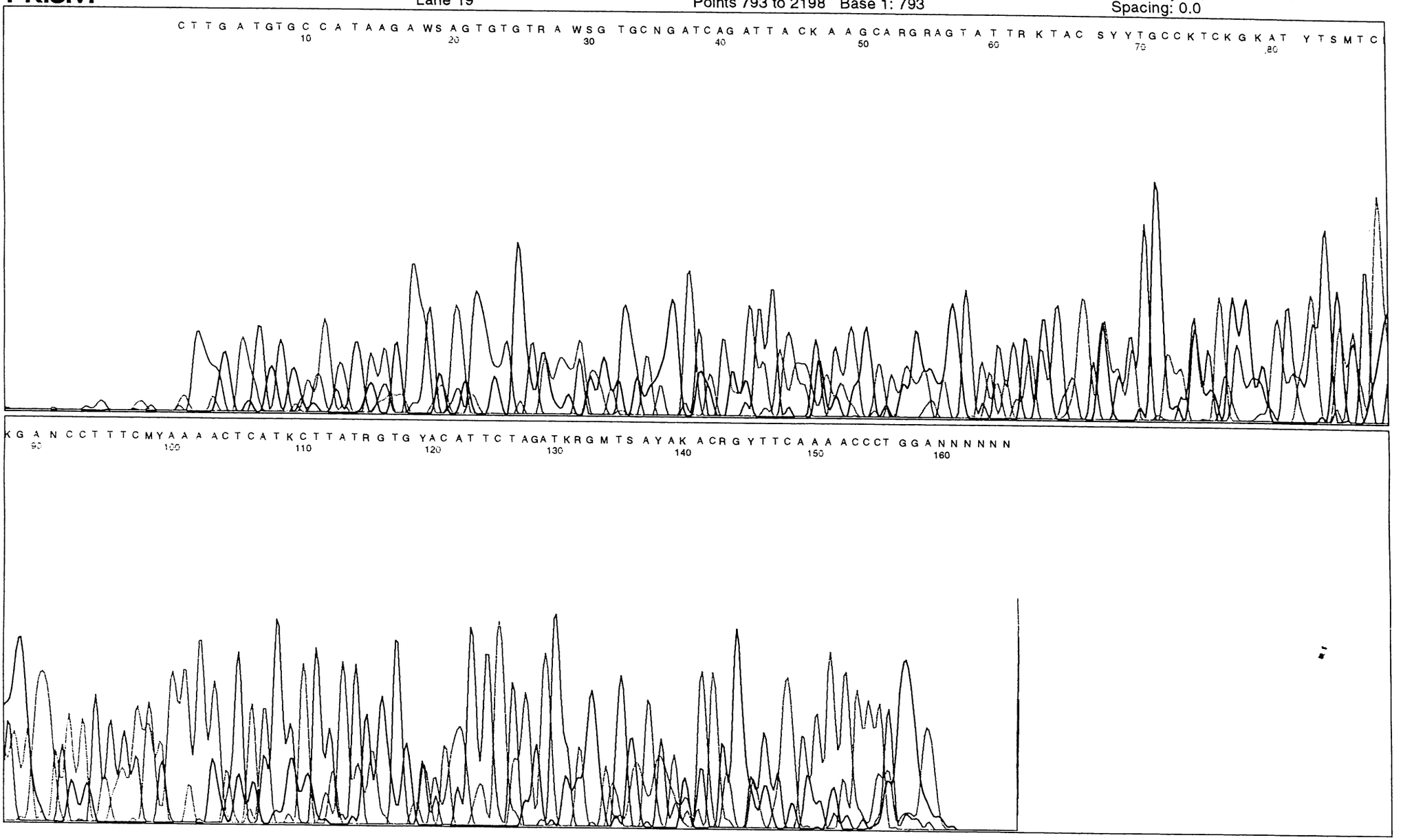
Tue, May 26, 1998 13:11
Spacing: 0.0



ABI PRISM

Model 377
Version 2.1.1

20•Mahlavu/TC148

Mahlavu/TC148
Lane 20

Signal G:479 A:587 T:417 C:357
DT4%Ac{A Set-AnyPrimer}
SN 569
Points 961 to 2356 Base 1: 961

Wed, May 27, 1998 08:22
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Spacing: 8.39 ABI200