ISAS - INTERNATIONAL SCHOOL
FOR ADVANCED STUDIES

Development of a transgenic animal model for investigating HCV induced autoimmunity

Thesis submitted for the Degree of

Doctor Philosophiae

Candidate: Lorenza Ciani

Supervisor: Prof. Francisco Ernesto Baralle

Academic Year 1997/1998

SISSA - SCUOLA
INTERNAZIONALE
SUPERIORE
DI STUDI AVANZATI

TRIESTE
Via Beirut 2-4

TRIESTE

ISAS - INTERNATIONAL SCHOOL
FOR ADVANCED STUDIES

Development of a transgenic animal model for investigating HCV induced autoimmunity

Thesis submitted for the Degree of

Doctor Philosophiae

Candidate: Lorenza Ciani

Supervisor: Prof. Francisco Ernesto Baralle

Academic Year 1997/1998

TRIESTE

ACKNOWLEDGEMENTS

The work described in this thesis was carried out at the International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste.

I thank my supervisor, Prof. F. E. Baralle, under whose supervision this work was carried out for his constant guidance and support throughout this project.

I am deeply indebted to Emanuele Buratti for his help, ideas, discussion and friendship throughout my time in this laboratory. A special thanks goes to Andres Muro for his timely advise and support, to Maurizio Romano for his help with computer programs and to Mauro Sturnega for his help in handling the mice.

I would like also to thank Prof. Stanta for his helpful assistence in the immunohistochemistry experiments.

I'm also grateful to the rest of my colleagues of the Molecular Pathology laboratory and ICGEB, in particular Gabriela Pittis, Fabiola Porro, Jorgelina Ottado, Marco Baralle, Martina Niksic, Michela Zotti and Rodolfo Garcia for their co-operation and friendship during these years. It has been really a pleasure to work daily with them.

Finally, I am grateful to Facundo for his constant moral support and to all my family for their encouragement and aid throughout.

TABLE OF CONTENTS

Chapter 3: Results

Chapter 1

Introduction

1.1 The discovery of the Hepatitis C Virus (HCV)

The hepatitis A and B viruses (HAV and HBV) were already known and characterised in the mid seventies. When diagnostic tests for both viruses were well in place they were applied to sera from patients with non-B hepatitis acquired after transfusion. Those cases which were not found caused by HAV were termed non-A, non-B hepatitis (NANB) (Freinstone et al., 1975). At the time non-A, non-B hepatitis accounted for as many as two thirds of transfusion-associated hepatitis cases. The agent and the disease of non-A, non-B hepatitis became the subject of intensive research.

The physicochemical properties of this agent suggested that it must be a virus (Bradley et al., 1983; Bradley et al., 1985) but nothing specific was known about it until 1989. In this year workers at the Chiron corporation (California) were able to clone a RNA genome that could correspond to a virus responsible for most of these posttransfusional hepatitis (Choo et al., 1989) . They started the isolation from large volume of sera from infected chimpanzees to obtain an infectious plasma pool. Nucleic acids exstracted from a crude viral pellet was used to generate a cDNA library that was screened with sera from a patient with postransfusional non-A, non-B hepatitis. A single plaque was identified and this positive plaque was then used as a hybridisation probe in a series of experiments which demonstrated that:

a) the cDNA hybridised to RNA from infected chimpanzee liver and serum ,

b) it did not hybridize to RNA from uninfected liver or to DNA from infected liver.

These results excluded the possibility that the cDNA insert could have derived from the host genome and proved the presence of an exogenous RNA molecule associate to NANB hepatitis infection.

The complete nucleotide sequence of the RNA genome of the original HCV isolate was then determined from overlapping clones and was termed epatitis C Virus (HCV). Thus, without specific knowledge of the type of virus involved, viral nucleic acid was cloned from infectious plasma samples. Even today the structure of the viral particle (Kaito et al., 1994; Myamoto et al., 1992) is poorly known due to the lack of an efficient cell colture system. In fact, this structure has been putatively identified only in relatively few studies using electron microscopy (Kaito et al., 1994). The structure shows a striking heterogeneity in density ranging from 1.03 to 1.72 g/ml (Yuasa et al., 1991) . This variability is due to their circulation either as free, infectious particle (low density) or in association with immunoglobulin (high density). Furthermore, the low density particles are frequently bound to lipoproteins (Thomssen et al., 1992).In any case, the knowledge of the RNA sequence and its homology to well known viral families, have allowed the prediction of the sequence of many of the viral proteins and hence the set up of immunological tests to detect Ab response to the viral infection.

Immunological or PCR-based assays to detect negative HCV-RNA molecules have indicated that this pathogen is the predominant cause of post transfusional NANB hepatitis around the world (Alter et al., 1989). Anti -HCV reactivity is associated with most community-acquired NANB hepatitis cases in the United States and Western Europe, and is a major cause of cryptogenic chronic liver disease in Italy (Colombo et al., 1989). The persistent infection of this virus commonly results in chronic active hepatitis which may lead to liver cirrhosis and hepatocellular carcinoma (Saito et al., 1990). Random screening of blood donor population has indicated that worlwide there could be as many as 500 million chronic carriers of the virus(Dillon and Dusheiko, 1995), confirming HCV as a major human pathogen.

The single most stricking feature of HCV is its extraordinary ability to evade host immune response and establish persistent infection in the majority of infected persons (Alter et al., 1992; Farci et al., 1994). Interestingly, this feature is also present in those patients which develop a specific humoral and cellular response against HCV proteins, highlighting the fact that even today very little is known concerning the natural course of HCV infection.

For this reason the aim of present day research has been predominantly focused on identifying the different viral and host factors that correlate with the progression of this disease.

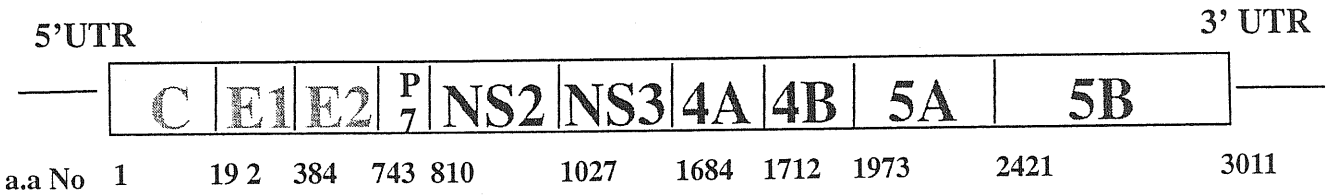
1.2 Structure of the HCV genome

The genomic organization of the HCV virus (Fig.1.1) , indicates that it is closely related both to pestiviruses and flaviviruses (family Flaviviridae) (Choo et al., 1991; Okamoto et al., 1991) This virus contains a positive-stranded RNA genome of approximately 9.400 nucleotides in length which is divided in three main regions. The first is the 5′ untranslated region (341 nucleotides long) which is able to form a stem-loop structure that precedes a single open reading frame (ORF) encoding for a precursor polyprotein of 3008-3037 aminoacids. This polyprotein is cotranslationally or posttranslationally cleaved into separate proteins by a combination of host and viral proteases (Grakoui et al., 1993). Table 1 summarizes the functions and the size of each of these viral proteins.

At the end of the carboxy-terminal region on the single HCV ORF there is a short 3′UTR (Han et al., 1991) which consists of approximately 50 nucleotides followed by a poly (U) tract, which in some isolates is replaced by a poly (A) tract. Recently, it has been discovered that these polynucleotide tracts do not represent the end of the HCV genome and that they are all followed by a highly conserved terminal sequence of approximately 100 nucleotides (Tanaka et al., 1996) which is believed to possess a complex and conserved secondary structure involved in protein binding .

Very little is known concerning HCV-RNA replication but the similar genetic organisation shared by HCV with flaviviruses and pestiviruses makes it likely that it includes the synthesis of negative strand intermediates which in turn drive synthesis of new positive RNA genomes (Fong et al., 1991). In fact, the recent detection of negative strand mRNA is consistent with this hypothesis of HCV RNA replication .

Similarly to what has been found in flavivirus and pestivirus also in the HCV virus the structural proteins are located in the N-terminal region of the genome whilst a variety of non structural proteins are located at the C terminus of the polyprotein (Tanaka et al., 1996).



STRUCTURAL PROTEINS:

Core= Nucleocapsid protein

E1= Envelope 1

E2= Envelope 2

NON STRUCTURAL PROTEINS:

NS3=Metallo protease,Serine protease, RNA elicase

4A= Cofactor

5B= Rna polymerase

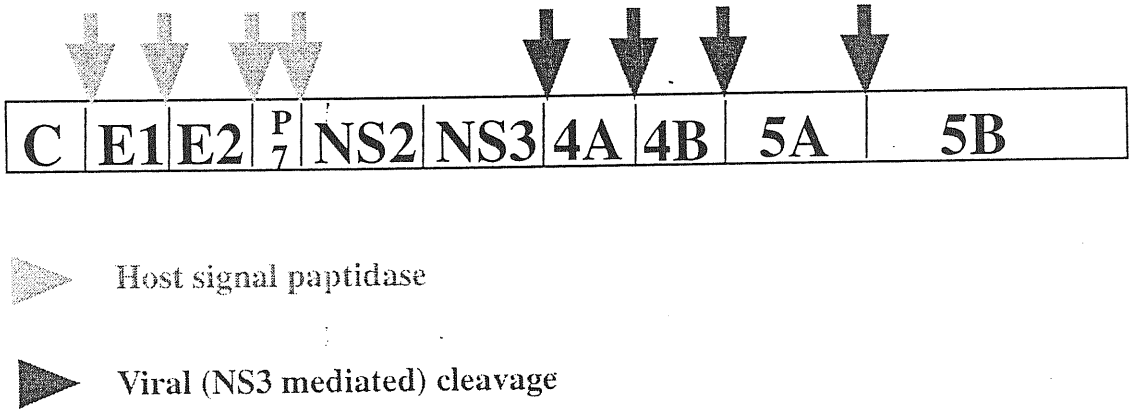


Fig.1.1 : Genomic organisation of the HCV RNA (upper panel)

Proteolytic processing of the HCV polyprotein (lower panel)

The structural region of HCV although shorter than that of flaviviruses and pestiviruses is organised in a similar fashion, with a basic N-terminal (p20) nucleocapsid Core protein (C) followed by two glycoproteins gp35 and gp70 . The gp35 protein (E1) probably corresponds to a matrix/envelope glycoprotein in the virion, whereas gp70 (E2) probably rapresents a second envelope glycoprotein. Both proteins are released from the precursor polyprotein by cellular proteases associated with the membranes of the endoplasmic reticulum (Hijikata et al., 1991). Although their exact function is unknown the fact that these two proteins are involved in the formation of complex protein-protein associations suggests a connection with the control of virus replication. (Dobuisson et al., 1994; Landfor et al., 1993). Finally, E1 and E2 are important in terms of antigen variation studies, E2 being the most variable region of the HCV genome (Kato et al., 1992; Weiner et al., 1991). These variations are assumed to be caused by random mutations, raising the possibility that the selection of such mutants allows the virus to escape the neutralising antibody response in the host.

Similarly to what has been observed for the structural proteins the non structural proteins are released from the precursor by successive proteolytic cleavages. The exact number of these non structural proteins in unknown but by comparison with the organization of flaviviruses they have been divided in NS2,NS3,NS4 (A/B) and NS5 (A/B) .

- NS2

NS2 is a transmembrane protein linked through its carboxy terminal region at the endoplasmic reticulum of the cell. Although NS2 is closely associated with the structural proteins the biological function of this protein is still unclear. In fact, it has recently become clear that the carboxy-terminus of this protein contributes significantly to the proteolytic cleavage occurring at the NS2/NS3 junction (see below). This region, together with the N-terminal region of NS3, forms a proteolytic domain which does not present any analogy with other classes of proteases and

which is localized in the carboxy terminal region. In particular, His-952 and Cys-993 in NS2 are the residues involved in catalytic activity of this enzyme (Hijikata et al., 1993b). It has been suggested that this cleavage is a pivotal step in the HCV virus replication (Hijikata et al., 1993a).

- NS3

NS3 is a protein of 70 KDa with many of biochemical functions, the principal being that of a serine protease. In fact, in its amino acidic sequence it is possible to find three aminoacids that characterise the catalytic triad of a typical serine protease (His-1083,Asp-1107 and Ser-1165) and for this reason NS3 is the protein most extensively studied of the whole HCV genome.

Several in vitro transcription/translation experiments and transient mammalian expression system have been used to observe the proteolytic activity of this viral protein (Bartenschlager et al., 1993; D'Souza et al., 1995; D'Souza et al., 1994; Eckart et al., 1993; Tomei et al., 1993) confirming that the protease activity of this protein is located in the amino-terminal region. NS3 is known to be responsible for the cleavage at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A, NS5A/NS5B junctions, thus obtaining the mature forms of the different proteins. It is very important also to note that this proteolytic activity needs some cofactors, the most important of which is the NS4A protein (Kim et al., 1996). These two proteins form a stable complex between each other (Tomei et al., 1993), a hypothesis confirmed by crystallographic studies (Bartenschlager et al., 1994). In addition, the sequence of the NS3 protein also reveals motives characteristic of NTPase and RNA helicase enzymatic motifs. Probably, the interplay between these different domains of NS3 represents a critical step in virus replication.

-NS4

The NS4 region of the HCV polyprotein contains two proteins, namely NS4A and NS4B. The NS4A protein is a small protein of 8 KDa in size. However, the C-

terminus of NS4A has been shown recently to be required (either in cis or in trans) for NS3-mediated clevage at the NS3/NS4A and NS4B/NS5A junction and to accelerate the NS3-mediated cleavage of the NS5A/NS5B site. (Failla et al., 1994)In addition it functions as anchorage of the replication complex(Failla et al., 1995). Currently, there is no ascribed function for the NS4B protein.

-NS5A/B

NS5A/B are two proteins which are obtained through the action of NS3 protease in conjunction with NS4A. Both proteins are hyperphosporilated (Kaneko et al., 1994) but the biological significance of this modification is still unclear. Recent work (Hwang et al., 1997) has suggested that NS5A/B could be a critical factor in determining the susceptibility of the virus to treatament with interferon (INF), probably through a direct interaction of NS5A with the INF-induced protein Kinase. The presence of the characteristic motive G-D-D- in the NS5 sequence suggest also the presence of a polymerase activity.

Table 1. HCV proteins

protein	aa size	function
Core	191	virus nucleocapsid
E1	192	glycoprotein
E2	327?	glycoprotein
NS2	316	metallo-proteinase
NS3	631	multifunctional protein(protease, helicase,NTPase)
NS4A	54	cofactorof NS3
NS4B	261	unknown
NS5A	448	replicase function?
NS5B	591	polymerase

1.3 The 5′ UTR

The 341 nucleotide region constituting the 5′ UTR of the Hepatitis C Virus is the most highly conserved region of the virus genome. It folds upon itself to form a complex secondary structure. (Tsukiyama-Kohara et al., 1992). In addition inside the 5′ UTR of different strains there are up to five AUG codons which are not used for initiation of translation (Reynolds et al., 1995).

Both characteristics closely resemble the situation found in picornaviruses, in which the process of initiation is governed by a cap indipendent mechanism which causes the direct binding between the ribosome and the viral RNA (Jakson et al., 1990). This kind of structures is called an Internal Ribosome Entry Site (IRES). Therefore, by analogy with the picornavirus it has been suggested that also the HCV 5′ UTR directs ribosome binding to an internal AUG translation initiation site (Brown et al., 1992; Jakson et al., 1990; Tsukiyama-Kohara et al., 1992; Wang et al., 1993). The first experiments in this sense were performed by Tsukiyama-Kohara (Tsukiyama-Kohara et al., 1992) which proved that in an in vitro system the 5′ UTR of HCV is able to start the translation in a Cap-independent manner.

There are two advantages for using this method of translation initiation. The ribosome bypasses highly stable secondary structures at the 5′ terminus of the viral genomes, which may be required for RNA replication and which would make a scanning mechanism very difficult to take place. It is also possible that the HCV virus have retained (or evolved) an IRES because it allows for regulation of translation in a fashion that promotes the long-term persistence of HCV infection in humans.

The first experimental model of the secondary structure of the HCV 5′UTR was proposed by Brown et al (Brown et al., 1992) and is shown in Fig1.2. In this work the authors proposed a thermodynamic and ribonuclease sensitivity analysis of the primary sequence of the 5′ UTR together with a comparison of the resulting prediction to the structure of two distantly related pestiviruses. Recently, the discovery of GBV-B virus whose genome is similar in length and organization to that

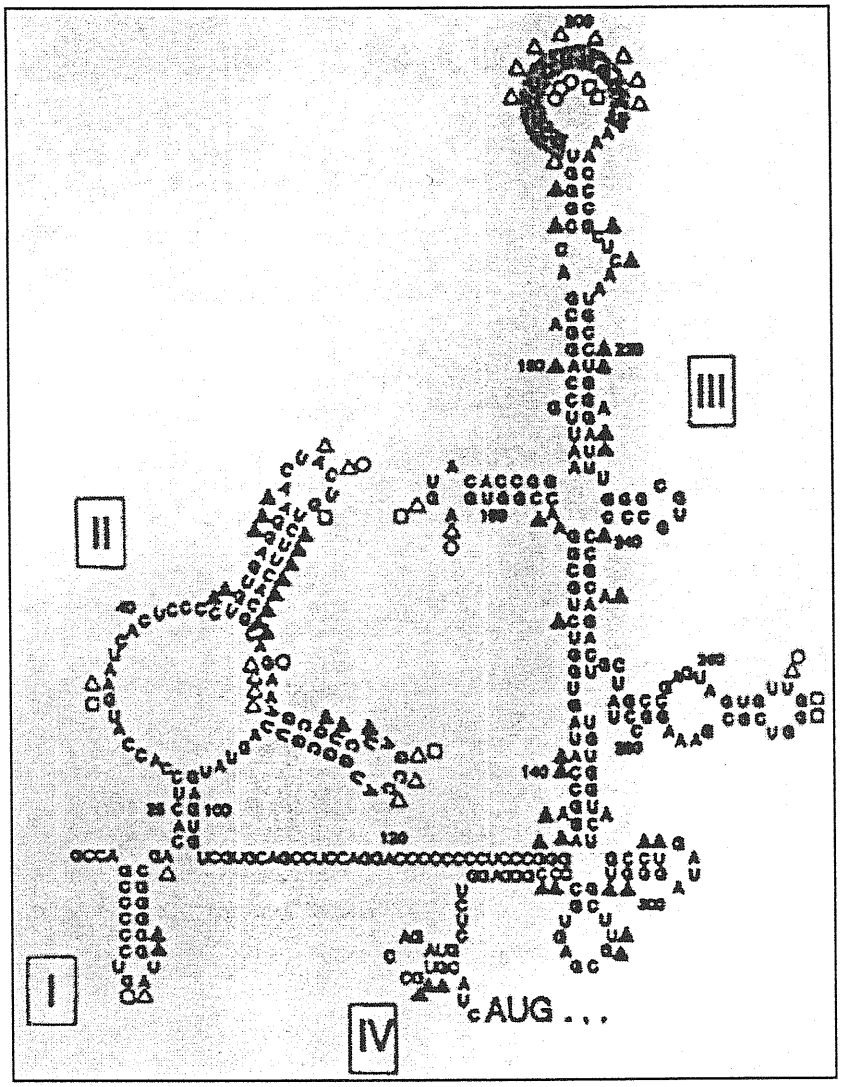


Fig 1.2 Secondary structure of the 5' UTR of the HCV proposed by Browm et al (1992). The shaded backround indicated the pyrimidine-rich tract within the loop of the domain III.

of HCV (Simmons et al., 1995) has provided additional data concerning the structure of the HCV 5′ UTR (Honda et al., 1996b) Currently, the stem-loop structures characterizing the HCV IRES have been termed domain I, II, III, and IV.

Fukushi et al (Fukushi et al., 1994)had already shown that almost the complete 5′UTR sequence containing the major stem-loop structures predicted by the study of Brown et al 1992 was necessary for optimal IRES activity, with the only possible exception being the most 5′ located hairpin structure (domain I) (Rijnbrand et al., 1995). Several additional studies performed by different laboratories have allowed to reach a consensus concerning the identification of the minimal region essential for IRES activity, and current opinion agrees that optimal IRES activity resides in a segment from nucleotides 40 to 370 of the HCV 5′UTR. Interestingly, since the starting AUG of the HCV polyprotein is at position 342, at least the first 12-30 nucleotides of the core protein coding region are part of the IRES element (Reynolds et al., 1995). The refined model of the secondary structure of HCV 5′ UTR proposed from Honda (Honda et al., 1996a) has shown that the initiator AUG is located within the single-stranded loop segment. This fact represents a fundamental difference between the HCV IRES element from picornaviral IRESs where the efficiency of internal initiation of translation is not influenced by sequences downstream of the initiating AUG codon (Honda et al., 1996a; Lu and Wimmer, 1996) However, it must be noted that this portion of the IRES, although important, is not essential for IRES activity since several investigators have found HCV IRES activity in the absence of the core coding region (Wang et al., 1993). The greatest stem-loop domain of HCV 5′UTR is the domain III, located between position 125 and 323, and capable of forming a large secondary structure characterised by multiple stable stem-loop and by the presence of different regions important in the regulation of transcription. These regions are a pyrimidine-rich tract (nt 191-199), a AUG triplet located 20 nucleotide downstream and a region (nt 192-203) complementary to bases 461-471 of human 18S RNA (Brown et al., 1992)

In addition to the secondary stem-loop structures several studies have also shown that more complex tertiary structures of the HCV 5'UTR such as a pseudo knot (Wang et al., 1995) or double-stranded region (Simmonds et al., 1993) are important for the maintenance of IRES function. In fact, mutations that destroy the stability of the pseudoknot structure or the double-stranded region strongly reduce the efficiency of cap-independent translation both in vitro and in vivo.

It is also very important to note that the secondary structure predictions for different variants of HCV are remarkably conserved. Therefore, the importance of maintaining a correct secondary and tertiary structure clearly reflects the fact that the process of translation initiation is necessarily dependent on the interaction between specific RNA sequences/structures and cellular/viral proteins. Therefore, several studies have been recently aimed at identifying some of these factors.

Two proteins of 87 and 120 kilodalton (named p87 and p120 respectively) have been observed to bind specifically domain III, but their identity is still unknown (Yen et al., 1995).

In addition, an association between protein binding and IRES activity has also been recently described to exist also for domain II (nucleotides 23 to 102), the other main stem-loop domain of HCV 5'UTR, with the identification of a 25 kdal protein binding specifically to one of its stem-loop elements. Mutational analysis has shown that this interaction is essential for correct IRES functioning .(Fukushi et al., 1997)

1.3.1 Viral RNA detection

Although the levels of viral RNA is not enough to allow detection using conventional Northern blots the use of RT-PCR techniques represents a very sensitive assay for detecting its presence in the blood stream and in tissue biopsy specimens.

The PCR assays to detect the presence of HCV RNA usually require the use of primers specific for the 5' UTR region, since this is the most highly conserved portion of all HCV isolates found to this date (Garson et al., 1990; Han et al., 1991; Okamoto

et al., 1991). However, the fact that the HCV 5′ UTR is also very similar to other 5′ UTR of pestiviral genomes (45% to 49%) it is advisable to design either the PCR primers or the hybridisation probes in regions that are not highly conserved with the pestiviral genomes, thus avoiding a potential misdiagnosis.

The usefulness of this technique is highlighted by the fact that viremia can be detected within only a few days of exposure to the virus, and many weeks before elevation of viral antibody level (Shimizu et al., 1990). Finally, this kind of assay allows not only the diagnosis of HCV infection in Chronic NANB hepatitis patients who may be seronegative in immunological tests, but also provides valuable information concerning the viremic status in infected patients when liver function is normal. Finally, PCR assays provide a useful tool to monitor therapeutic efficacy.

1.3.2 HCV-RNA genetic variability

HCV-RNA shows significant genetic variability, with an estimated rate of nucleotide change of approximately 103 substitutions per site per year (Simmons et al., 1995). This genetic variability can be seen in all domains of HCV-RNA but it significantly predominates for the E2 envelope protein, and in particular its 5′ a domain (also called HVR1).

As already discussed in a previous chapter the most conserved portion of the HCV genome is represented by the 5′ UTR region. Therefore the sequence variability observed in this region has been used to classify the viral isolates in six different genotype (1,2 and 3 representing the major genotypes, whilst 4,5 and 6 the remaining types.).

Although determination of the complete 5′UTR nucleotide sequence is the most reliable method for correctly identifying different genotypes of HCV this kind of approach is not very practical for large studies. Therefore, many of the published method of genotyping are based on the amplification of partial viral sequences, either using type specific primers that selectively amplify different genotypes, or by

hybridisation of the PCR product with genotype-specific probes coupled by restriction fragment length polymorphism.

Interestingly, some genotypes of HCV (types 1a,1b,2a and 2b) show a broad world-wide distribution, whereas others, (such as type 5a and 6a) are only found in specific geographical regions. For example HCV subtype 1b frequency is high in Japan and Europe but lower in the United States, where HCV subtype 1a is more prevalent. Some genotypes are predominantly found outside these areas, such as type 4 in Central Africa and the Middle East,(Xu et al., 1994) type 5 in South Africa and type 6 in Singapore (Fig 1.3). Finally, it has also been reported that the prevalence of different genotypes may change with age (i.e., in Europe genotype 3a is more common in young individuals than 1b). In addition, the presence of subtype 1b in posttransfusion and chronic hepatitis and subtype 3 and 2 in intravenous drug users has also been reported (Pawlotsky et al., 1994; Pol et al., 1994). The reason for all these differences is still largely unknown, but these kind of studies have found their reason in trying to correlate infection by a particular genotype and clinical course of the disease.

1.3.3. Pathogenesis of HCV: genotype dependent?

An attractive area of research into HCV is the investigation of possible differences in the course the of disease associated with different genotypes, such as the rate of development of cirrhosis and hepatocellular carcinoma, and whether certain genotypes are more or less likely to respond to interferon treatment. However, it is important to bear in mind that any correlation between genotype and illness progression is also complicated by the necessity to take in account a myriad of other factors, such age and duration of the infection.

Nonetheless, an association between chronic infection has been observed for genotype 1b (Dusheiko et al., 1992; Pistello et al., 1994; Silini et al., 1995). Infection by this genotype also seems to be correlated by the presence of severe chronic hepatitis and cirrhosis although conflicting data have been reported on this issue. (Yamada et

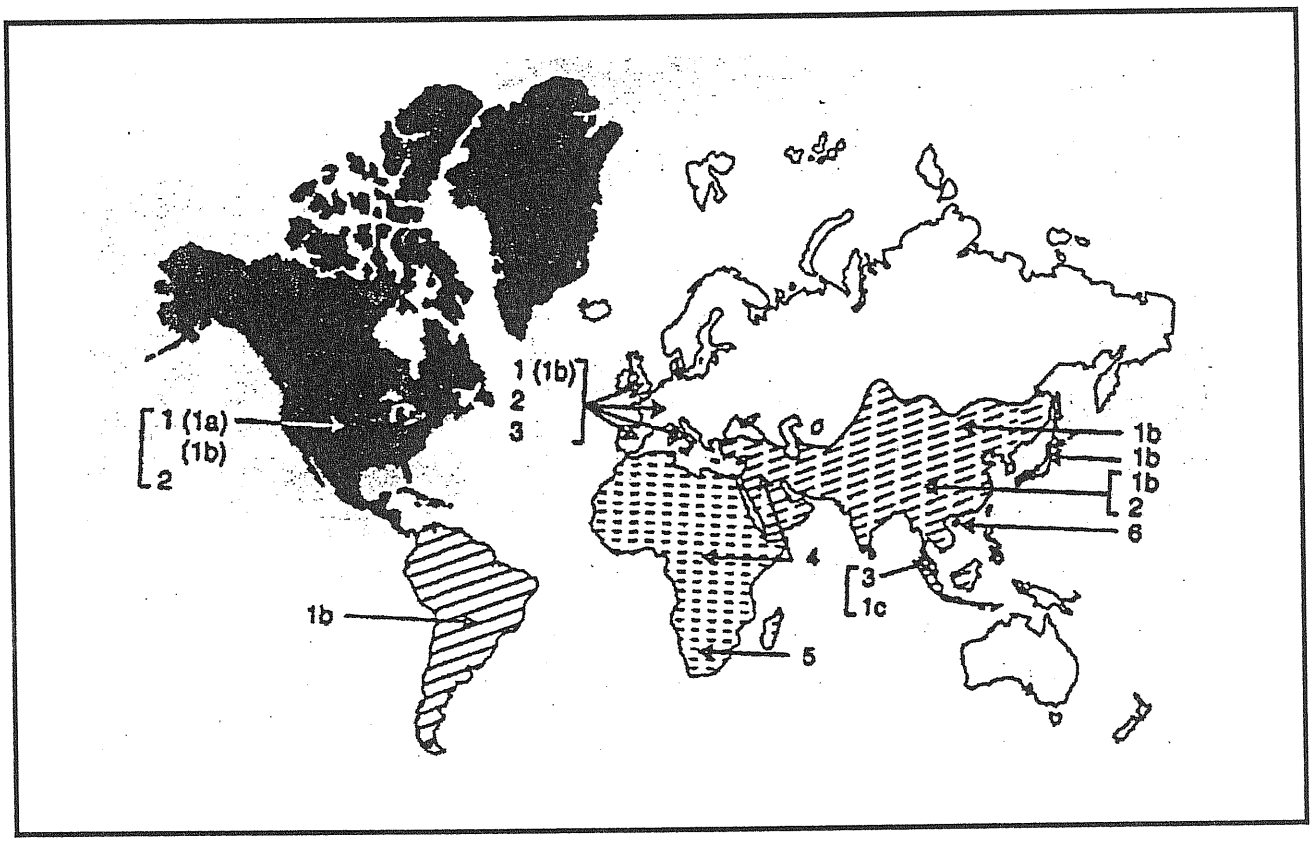


Fig. 1.3 Geographical distribution of HCV genotype and subtype

al., 1994). Recently, a study based on a large population of patients from Italy and France has confirmed the association of subtype 1b with the presence of cirrhosis and, most importantly, a correlation between infection by subtype 1b and long-term duration of HCV infection. Concomitantly to these studies it has also been possible to determine that a poor response to INF treatment is associated in particular with subtype 1b infection, whilst a stronger response to INF therapy was observed in genotype 3 infection

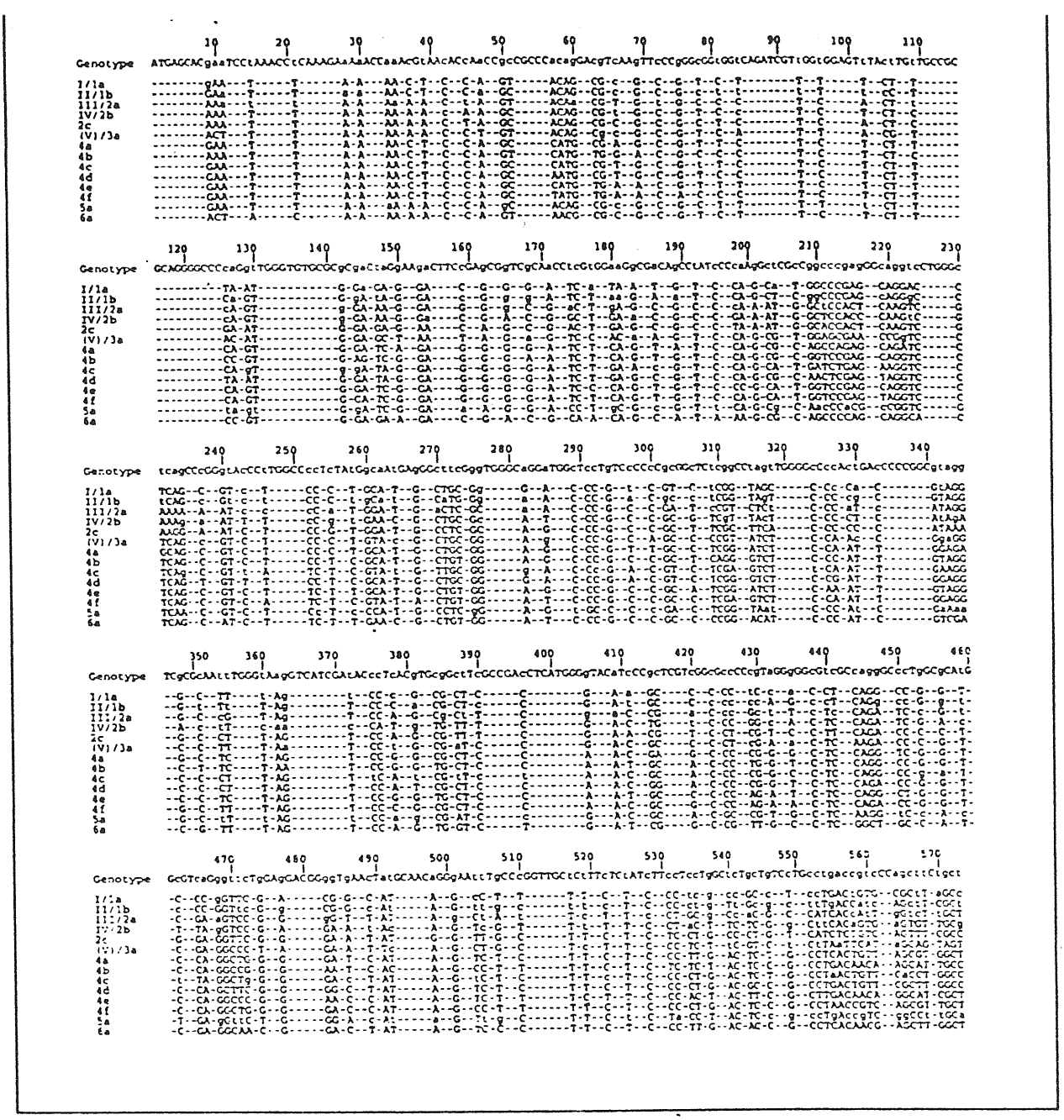
Until now very little is known concerning the reason for these differences mainly because very little is known concerning which HCV proteins might influence the rate of replication, cytopathology, and resistance to anti viral treatment. There may be differences in the activity of viral proteinases in processing the HCV polyprotein after translation or in the activities of proteins involved in RNA replication, leading to alteration in the host response. Variation in the envelope proteins of the different genotypes may contribute to differences in cell tropism and the extent to which infection causes cytophathic changes in the cell during the latter stages of virus assembly and release. In addition, the variables and hypervariable regions observed in different HCV genomes suggest an ability of the virus to evade immune surveillance by rapid mutation, as has been observed in other viruses (Payne et al., 1987). Alternatively, it could be also important consider the different ability of the 5′ UTR belongs to different HCV genotype to promote the translation. In fact evidences exist (Buratti et al., 1997) suggesting that the 5′ UTR of genotype 3 has a lower translation efficiency when compared with the 5′ UTR of genotype 1 and 2.

1.4 The HCV core protein

The HCV core protein coding region is situated at the amino terminus of the polyprotein, it is highly basic in nature and is considered to be the most likely viral capsid protein. In general, this protein is highly conserved between the different HCV isolates as was immediately apparent by initial studies in which the nucleotide and aminoacid sequence of different HCV Core proteins were compared (Fig 1.4). In fact, the results of these studies have suggested a range of 85.3-100% of aminoacid identity between the different HCV strains. Moreover, this protein contains many proline residues (Collett et al., 1988) which are likely to be important in maintaining the secondary structure. In addition, approximately 16% of the aminoacid composition is constituted by lysine and arginine residues which are located in three clusters (aa 6-23, 39-74 and 101-121). (Shih et al., 1993). In particular, the arginine and lysine residues located between residues 39 to 74 are highly conserved, suggesting that this domain may represent and important RNA-binding site. The remainder of Core protein is hydrophobic in nature, and it is probably these regions that are involved in protein-protein or protein-RNA interaction.

A key feature of the HCV core protein is its characteristic to undergo a complex maturation process to yield several truncated forms. Initially, an immature Core protein (191 aa in size, also known as P21) is produced after cleavage of the polyprotein precursor by host signal peptidases located inside the ER at the N terminus of the E1 sequence. This form of the Core protein is predominantly associated with the ER (Santolini et al., 1994) and this association is mediated by the presence of two distinct hydrophobic regions (residues 121-151 and 170-191). The P21 protein then undergoes a second proteolytic event to produce a mature polypeptide of 173 aa in size (also referred to as P19) which, although associated with the ER just like P21, can also be translocated into the cytoplasm (Santolini et al., 1994; Selby et al., 1993) and possibly into the nucleus (Landfor et al., 1993; Lo et al., 1995; Shih et al., 1993). Therefore, this second cleavage event represents a potentially key step in the viral life-cycle, allowing a change in cellular compartment localisation

A



B

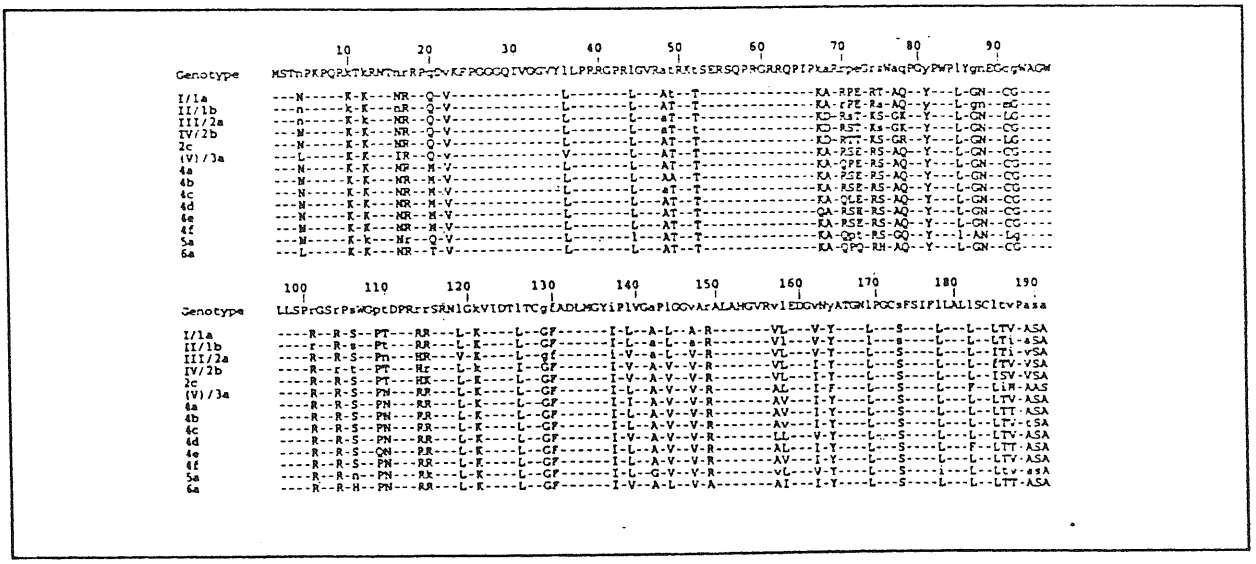


Fig 1.4 Aligment of the consensus sequences (A) and of the deduced aminoacid sequences (B) of the C gene belonges to different genotype of HCV.

of this protein. This cleavage event may be regulated in a preferential and sequential manner since it is already known to be preceded by that at the 191 residue. Although the exact regulation of this event is not well understood, the most likely mechanism of action is that cleavage at residue 191 is required to expose the cleavage site at residue 173. (Harada et al., 1991; Hijikata et al., 1993a; Santolini et al., 1994).

An important observation has been the presence of specific protein-protein interaction of the different forms of core protein which appear to require a specific aminoacid sequence that is very conserved among different HCV isolates (Bukh, 1994). Matsumoto et al(Matsumoto et al., 1996) also showed that these multimers can form both in vitro and in vivo. The homotypic interacting domain has been tentatively mapped around the 50 aminoacids that overlap the ribosome binding site and the RNA binding domain of the protein (Santolini et al., 1994). It is not clear whether this partial overlapping may have any functional significance, but it is possible that the multimerization of the Core protein may alter the conformation of the RNA-binding domain in order to facilitate RNA-protein interactions in virus assembly.

Recently, (Liu et al., 1997) have proposed an interesting theory that correlates this complex Core protein maturation process with the events that occur during virus replication. They suggest that the possible association between P21 and P19 on the ER in the cytoplasm is the driving force keeping the P19 sequestered within the ER and that eventually the amount of P21 being produced is insufficient to keep the P19 form from diffusing in the cytoplasm and the nucleus. The cytoplasmic localisation may then function as a scaffold for viral assembly and genomic RNA packaging, whilst the entry in the nucleus may serve to regulate gene expression. In keeping with this it has been recently observed that the core protein is able to suppress apoptotic cell death in primary rat embryo fibroblasts (Ray et al., 1996) and that it can also specifically interact with the cytoplasmic tail of the Lymphotoxin-b receptor a member of the tumor necrosis factor receptor family.(Matsumoto et al., 1997)

The whole issue of elucidating the core protein biological function is also complicated by the fact that in addition to P21 and P19 another truncated form (P16) has also been described (Lo et al., 1994). The P16 protein consists of about 151 aa and is apparently co-amino terminal with the full length core protein of 191 aa. Characteristically, it shows an exclusive nuclear localisation with enrichment in nucleoli (Lo et al., 1995). Interestingly, the amount of this protein in the different strains of HCV has been observed to be very variable. This is due to the fact that production of this truncated form depends from the presence of a Lysine residue at position 9 in the core gene sequence. Although the biological functions of P16 remain unclear its exclusive localisation in the nucleus and nucleolus suggests that it may have biological functions very different from those of P21 and P19. (Lo et al., 1995).

In conclusion, the complexity of events that surround the core protein maturation process clearly indicates its critical role in the viral life-cycle.

An important finding regarding the biological function of the core protein has been its association with the lipid droplets present in abundance in the liver of infected animals (Barba et al., 1997). This is in keeping with the fact that the HCV virus had been previously described as a lipid-containing virus and in plasma patients shows an heterogeneous density distribution partially due to the binding to low density lipoprotein, very low density lipo protein, IgG ,IgM and high density lipoproteins. Considering that a characteristic of HCV infection is the presence of liver steatosis it is plausible that this condition could arise at least in part from direct effects of HCV proteins on lipid metabolism.

1.4.1 The nuclear localisation of the HCV Core protein

Particularly interesting is the still disputed nuclear localisation of the core protein. The possibility for the core protein to be translocated in the nucleus was first suggested by the presence in its sequence of three nuclear localisation signals that resemble those already described for other nuclear proteins (Dingwall and Laskey, 1986) at residues 38 to 43, 58 to 64 and 66 to 71. In addition, the presence of a putative

DNA-binding motif supported the hypothesis that this protein could be also function as a gene-regulatory protein (also in consideration of the fact that it is probably post-translationally modified through phosphorylation) (Landfor et al., 1993).

The nuclear localisation was first observed by Lo S-Y et al (Lo et al., 1995) in immunofluorescence experiments and more recently by Ray RB et al. (Ray et al., 1996) who showed that HCV Core protein in immortalised cells or in cells recovered from mouse tumor was present as granular inclusion bodies in the nucleus. In addition, nuclear staining of the Core protein was also observed in liver tissue from biopsy samples of patients with chronic HCV infection (Suzuki et al., 1995).

However, the issue is still undecided because other recent works demonstrated that in tissue sections HCV antigens could be exclusively detected in the cytoplasm of the infected hepatocytes, whereas the nuclei were never stained (Hiramatsu et al., 1992; Sansonno and Dammacco, 1993). In addition, (Barba et al., 1997) have observed the presence of the Core protein only as cytoplasmic protein using immunofluorescent techniques and scanning electron microscopy, suggesting that the nuclear localisation observed in previous works was an artefact caused by the localisation of the core protein on the outer surface of the perinuclear membrane.

1.4. 2 Core protein and immunity

The mechanisms responsible for HCV persistence and disease pathogenesis are not yet fully understood although it is likely that both direct (virus induced) and indirect (immunologically mediated) mechanisms play an important role.

Prospective studies on the immune responses to HCV have shown that antibodies to the viral nucleoprotein usually appear within 10 weeks from clinical onset. A transient IgM anti-core response has been clearly documented in many patients with acute post-transfusional hepatitis C and in patients with acute exacerbation of their chronic liver disease (Chen et al., 1992). Using epitope mapping techniques two apparently distinct B cell epitopes have been localised within the amino terminal end of the HCV nucleoprotein, a fact confirmed both by using

recombinant proteins and synthetic peptides (Nasoff et al., 1991; Okamoto et al., 1992).

Recently, it has been also observed that the Core protein can induce a vigorous Th-cell proliferative responses in patients with chronic HCV infection(Botarelli et al., 1993). Thus, a characterization of the cellular and humoral response to the core region would be extremely useful in defining the role of HCV nucleoprotein in host-virus interactions and above all, to develop more effective strategies for the prevention and treatment of this insidious disease.

1.4.3 HCV Core protein and Autoimmunity

A variety of genetic and immunoregulatory factors are likely determinants of either persistent hepatic infection or expression of an autoimmune disease which may occur secondary to persistent viremia. In fact, although NANB hepatitis is a disease of infectious aetiology, the patients can develop autoantibodies following infection with HCV. This HCV-associated autoimmunity is evidenciated by a broad spectrum of autoimmune diseases such a long-lasting inflammation, elevated levels of serum immunoglobulins or positive rheumatoid reaction. These patients normally respond to immunosuppressive therapy such as steroids and azathioprine. They are also partially resistant to anti-viral treatment such as interferons that in some cases can precipitate or exacerbate autoimmune disease symptoms. Recently, many studies have been focused on finding possible connections between HCV infection and induction of auto immune disorders.

The most likely candidate identified so far is a class of antibodies which cross-react between the HCV Core protein and the host derived gene product glyoxilate-oxidoreductase (GOR) (Mishiro et al., 1990; Mishiro et al., 1991). The presence in these patients of cross-reactive antibodies against both sequences is possibly associated with an auto-immune hepatitis type (Magrin et al., 1992; Michel et al., 1992). In particular, these studies have indicated that the cross-reactivity can be ascribed to the sequence RKTKRNTNR at the residues 9-17 of the Core protein which

is recognised by most HCV-infected humans (Kotwal et al., 1992; Okamoto et al., 1990; Sallberg et al., 1992) and which holds a 63% homology with the part of the GOR protein QKAKSNPNR at residues 19-27.

In conclusion, HCV- induced auto immunity may develop as a result of a molecular mimicry (Oldstone, 1987), in which an immune response mounted by the host against a specific determinant of an infecting agent may cross-react with the mimicked host sequence, leading to auto immunity and in some cases to tissue injury and disease.

Fig 1.5 shows the possible association between HCV infection and autoimmunity.

1.5 Development of transgenic animals

Since the chimpanzee is the only non-human model which supports the replication of the HCV virus the development and use of transgenic mouse models represent the best way to study the production, transport, assembly and toxicity of HCV protein in liver cells. The hope is that through the use of effective models researchers will be able to learn how each of the HCV structural proteins behave within the hepatocyte, and whether these proteins can modify hepatocyte functions sufficiently to result in the acquisition of the ultra structural features of HCV-infected hepatocytes, and eventually their ability to kill the hepatocytes directly.

Several animal models have already been developed that express the HCV Core protein (Pasquinelli et al., 1997) and all the structural protein (Core, E1,E2) in the liver (Kawamura et al., 1997). Although all these animals were seen to express the full length of these proteins in the cytoplasm of their hepatocytes, the histological and biochemical evidence of liver disease or hepatocellular carcinoma have not been found in either case, suggesting that probably the HCV structural proteins are not directly cytophatic (Koike et al., 1995). On the other hand, a recent work of Moriya et al (Moriya et al., 1997) indicates that the stable and long term expression of the Core protein in the liver of transgenic animals may induce the development of progressive

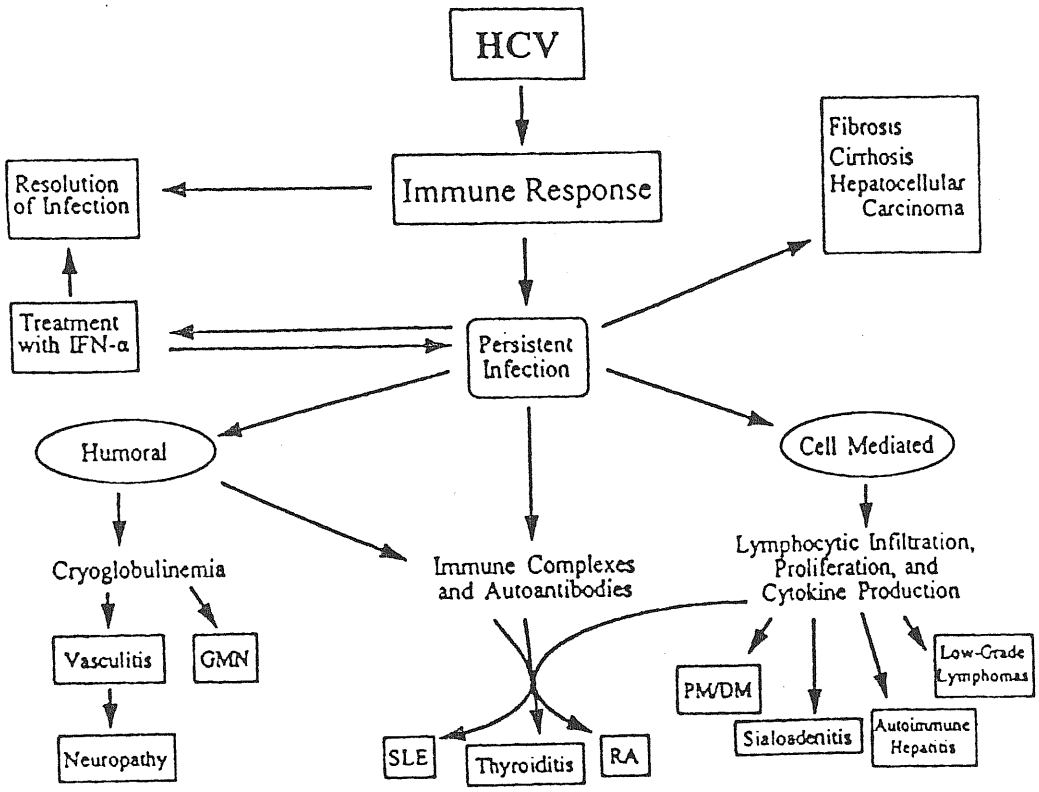


Fig 1.5 Scheme for HCV-associated autoimmunity. The immune/autoimmune perturbation have been emphasized

steatosis in the liver analogously to the characteristic feature of chronic hepatitis C. In addition, a recent work of Koike et al shows a strong relation between the chronic HCV infection and the development of an exocrinophathy involving the salivary and lachrymal glands that resembles the Sjogren syndrome. (Koike et al., 1997)

However, it must be pointed out that in all these transgenic animals the HCV proteins expressed were, necessarily, recognised as "self" by the host organism. Therefore, none of them would have been useful to clarify the role of the humoral and cellular immunological in the pathogenesis of this virus.

Aim of the project

Several lines of evidence have suggested that the pathogenic effects observed during HCV infection is mediated through the cytopathic effect of HCV encoded proteins and the immune response that they elicit.

In particular, antibodies have been described which are thought to be cross-reactive between the hepatitis C virus Core protein and a probable Host-derived gene product (GOR). The clinical significance of these antibodies lies in their possible association with autoimmune hepatitis type.

In order to clarify the mechanisms involved in HCV infection we have tried to develop a realistic animal model of HCV pathogenesis through the use of a tight inducible animal model in which the HCV Core protein is not to be expressed until it is triggered by an infection. For this, we have constructed a transgenic mouse for the Core protein (or selected viral protein) that can be induced through the Cre/lox system via an adenovirus infection.

Chapter 2

Materials and Methods

2.1 MATERIALS

2.1.1 Chemical reagents

Chemical were purchased from Sigma Chemical Ltd. and Carlo Erba Pharmaceutical and were of Technical or Analar grade.

2.1.2 Enzymes

Restriction endonucleases were supplied by Pharmacia, New England Biolabs Inc. and Boehringer Mannheim and were used according to the manufacturer's instructions.

T4 DNA ligase, DNA Polymerase I large fragment (klenow enzyme), Calf intestinal Phosphatase, T4 Polynucleotide Kinase were purchased from New England Biolabs Inc. and used according to manufacturers instruction.

Taq DNA polymerase was purchased by Boehringer Mannheim and were used as indicated in each reaction.

RNAase A was purchased from Sigma Chemicals Ltd. A 10 mg/ml solution of RNAase A was prepared in 5 mM EDTA and boiled for 10 minutes to destroy trace amounts of DNAase activity.

Proteinase K was purchased from Sigma Chemicals Ltd. in a 10 mg/ml solution.

2.1.3 Radioactive isotopes

Radioactive α-^{32}P dCTP, γ-^{32}P dATP and ^{35}S were supplied by Amersham U.K. Ltd.

2.1.4 Bacterial strains

Strains of the K12 E. Coli family were maintained in short term as single colonies on agar plates at 4°C and for longer term storage were kept on glycerol stocks, made by adding sterile glycerol to a final 15% v/v an overnight culture of bacteria grown in LB. Glycerol stocks were stored at -20°C.

The DH5α strain was used for transformation by plasmid and growth of the plasmid.

2.1.4 Liquid media

LB: Luria-Bertani medium per litre:

Difco Bactotryptone 10 g, Oxoid yeast extract 5 g, NaCl 10 g, pH 7.5.

Bacterial growth media were sterilised before using by autoclaving. Where appropriate antibiotics, ampicillin and tetracycline, were added to media to a final concentration of 75 μg/ml (ampicillin) and 50 μg/ml (tetracycline).

DMEM (Flow) culture media was used supplemented with 10% Foetal Calf Serum, glutammin 2 mM and gentamicin.

2.1.5 Standard solutions

All solutions have been defined in the text where first used except for:

TE: 10 mM Tris-HCl pH 7.4, 1 mM EDTA pH 7.4

PBS: NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, pH 7.4

10x TBE: 108 g/l tris, 55g/l boric acid, 9.3 g/l EDTA

5x Ficoll loading buffer: 0.25% w/v bromophenol blue, 15% w/v Ficoll type 400 in H_2O.

10x MOPS: 200 mM MOPS, 50 mM CH_3COONa, 10 mM EDTA pH 7.0

Formamide dye: 0.25% bromophenol blue, 0.25% xylene cianol, 20 mM EDTA in deionised Formamide

2.2 METHODS

2.2.1 Nucleic Acids Preparations

2.2.1.1 General techniques for nucleic acid purification and concentration

RNA and DNA were precipitated from aqueous solution containing 0.3M sodium acetate by adding ethanol, as described in Sambrook et al. (Sambrook,1989). The nucleic acid pellet was then resuspended in dH_2O at the required concentration. Protein contaminants were removed from aqueous DNA solutions by phenol-chloroform extraction (Sambrook , 1989).

2.2.1.2 Small scale preparation of plasmid DNA from bacterial cultures

Rapid purification of small amounts of recombinant plasmid DNA was basically performed the method described in Sambrook et al. (Sambrook,1989) based on alkaline lysis of recombinant bacteria. The final pellet was resuspended in 25 µl of dH_2O and 3 µl of such preparation were routinely taken for analysis by restriction enzyme digests.

2.2.1.3 Large scale preparations of plasmid DNA from bacterial cultures

Large scale preparations of plasmid DNA were carried out utilising the Jetstar plasmid kits by Genomed according to the manufacture protocol.This method yielded 2 µg-500µg of DNA depending on the plasmid grown.

2.2.1.4 Preparation of genomic DNA from tissues

Preparation of genomic DNA from tissue was carried out as described in (Laird et al., 1991) . 0.5 ml of lysis buffer were added to the tissues, Digestion is complete within several hours at 56 C with agitation.

Lysis buffer: 100 mM Tris HCL pH 8.5, 5mM EDTA, 0.2% SDS, 200 mM NaCl, 100 µg proteinase K/ml.

One volume of isopropanol is added to the lysate and the samples were mixed or swirled until precipitation is complete. Excess liquid was dabbed off and the DNA was dispersed in 200 µl of water. Complete dissolution of the DNA may require several hours of incubation at 37 C.

2.2.1.5 Preparation of RNA from tissues

To prepare RNA from tissues the basic protocol was followed (Sambrook, 1989). Frozen tissue (0.5-1 g) was homogenised in a correspondent volume (0.5-1 ml)

of D solution (Guanidine tiocianate 4M,β-mercaptoethanol 100mM, Na-citrate 25 mM,Lauroylsarcosine 0.5%) using a UltraTurrax homogenise. A further phenol-chloroform step was added to the basic protocol to improve the sample purification from proteins and fatty acids. The final pellet was then resuspended in 100 µl of ddH_2O and frozen at -80°C.

2.2.2 Estimation Of Nucleic Acid Concentration

2.2.2.1 Spectrophotometric

An optical density of 1.0 at 260 nm is roughly equivalent to a concentration of 50 µg/µl for double stranded DNA, 33 µg/µl for single stranded DNA and 40 µg/µl for RNA samples. the ratio of values for optical densities measured at 260 nm and 280 nm is 1.8 for pure sample of DNA and 2 for RNA, these are reduced by protein contaminants and therefore the values were used to asses both the concentration and the purity of the samples.

2.2.2.2 UV fluorescence of intercalated ethidium bromide

Nucleic acids size-fractionated on agarose or polyacrylamide gel electrophoresis can be visualised after staining with ethidium bromide (0.5 mg/ml) since fluorescence of ethidium bromide is enhanced by intercalation between bases in the nucleic acid helix. It is therefore possible to estimate the quantity of nucleic acid on a gel by comparing the intensity of UV-induced fluorescence of the sample with that of a standard sample of known quantity.

2.2.3 Electrophoresis Of Nucleic Acids

2.2.3.1 Agarose gels

RNA or DNA restriction fragments were size fractionated by electrophoresis in 0.8-1.8% w/v agarose gels containing ethidium bromide and 1x TBE. Horizontal 10x8 cm mini-gels were used routinely for fast analysis of DNA restriction enzyme

digests, estimation of amount of DNA, or DNA fragment separation prior to elution from the gel. Sample of up to 20 µl containing 1x Ficoll dye were loaded into submerged wells. These were electrophoresed at 50-80 mA for a time depending on the fragment length expected and the gel concentration, in a running buffer of 1x TBE. DNA was then visualised by UV transillumination (the lower limit for this was about 10 ng DNA) and the result could then be recorded by Polaroid photography.

RNA was also visualised in 1.5% agarose gels containing EtBr, 1x MOPS buffer, and 2% formamide. Samples containing 1x Formamide dye were loaded into submerged wells. These were electrophoresed at 80 mA for 20 minutes in a running buffer 1x MOPS. RNA was then visualised by UV transillumination. The total RNA quality and quantity was deduced from the ribosomal 28S and 18S RNA bands that are clearly visible while the mRNA is not.

2.2.3.2 Elution and purification of DNA fragments from agarose gels

This protocol was used to purify small amounts (less than 1 µg) of DNA for sub cloning or radiolabelling. The DNA samples were loaded onto an agarose minigel and electrophoresed as described above.

The DNA was visualised with UV light and the required DNA fragment band was excised from the gel. This slab was put into the minimum length of dialysis tubing with as little gel buffer as possible and was electrophoresed in a minigel apparatus at 150 V for the time required for the DNA sample to migrate out of the gel slice. At this stage the current was reversed for 30 seconds to elute any bound DNA from the dialysis tubing.

Buffer containing DNA was removed to a microcentrifuge tube and the tubing was washed twice with 100 µl of gel buffer. Aliquots were pooled and DNA recovered by ethanol precipitation. To remove small gel fragments remaining the aqueous solution was centrifuged for 10 minutes at 4°C and the supernatant was transferred to a fresh tube.

2.2.4 Identification and Analysis of Specific DNA Sequences

2.2.4.1 Detection of Specific DNA Sequences by Southern Blotting

Identification of specific DNA sequences cloned or specific products of PCR amplification was performed using the method originally described by Southern (Southern, 1975). The samples were electrophoresed as described previously. The gel was then washed in denaturing and neutralising solutions and the DNA transferred on a nitrocellulose membrane as described (Sambrook , 1989).

a) Hybridisation and prehybridisation of filter bound nucleic acids

Filters were enclosed in plastic envelopes and were incubated for 1-2 hours with moderate agitation at 65°C (DNA probes above 100 nucleotides) or at 42°C (oligonucleotide probes), in approximately 10 ml of hybridisation buffer (SET 5x, lyophilised milk 2%.) For hybridisation most of the solution was removed from the filters and denatured radioactively labelled probe was added and incubated, shaking, overnight at 65°C or 42°C. Probes were denatured by boiling for 5 minutes and were quickly cooled on ice.

b) Removal of non-specifically hybridised probe from Southern blot

Non-specific hybridisation of probes to the filters was reduced by multiple washes at decreasing salt concentrations as required. The following protocol was used:

a) 20 minutes at 65°C or 42°C in 3x SET, 0.5% SDS.

b) 20 minutes at 65°C or 42°C in 2x SET, 0.3% SDS.

c) 20 minutes at 65°C or 42°C in 1x SET, 0.1% SDS.

The filter was then dried and exposed using X-O-Mat "S" film (Kodak) at room temperature or at -80°C with an enhancer screen for the required time.

For the identification of a specific genomic DNA fragments, the genomic DNA extracted as described previously in the section 2.2.1.4 was digested with the opportune restriction enzyme over night at 37 C.

The digested DNA was checked on a 0.6% agarose gel and transferred on a nytrocellulase membrane as described previously.

Filters were prehybridised for 1 or 2 hours at 65 C in moderate agitation in prehybridisation buffer (SSC 6x, Denhart's 5x, Salmon Sperm 100 µg/ml, SDS 0.5%). For hybridisation most of the solution was removed from the filters and denatured radioactively labelled probe was added and incubated, shaking, overnight at 65°C . Removal of non-specifically hybridised probe from Southern blot was performed using different decreasing salt concentration solution as required.

2.2.4.2 Northern blot analysis

The total RNA extracted form the animals tissue was electrophoreses as described previously and transferred on a nylon membrane (Hybond N, Amersham).

The prehybridisation and hybridisation procedure is the same of that described for the genomic southern blot. In addition, the presence of 40% v/v of formamide in the prehybridisation and hybridisation solution, allow to decrease the hybridisation temperature..

2.2.4.3 Sequence determination by chain termination

Plasmidic DNA has been sequenced using the "T7 Pharmacia Sequencing Kit" supplied by Pharmacia Inc. based on "dideoxy chain termination" method previously published by Sanger et al. (Sanger et al., 1980). As template we used 10 µl from the mini preparation of plasmidic DNA. As primer we used the Bls polylinker universal and reverse primer and specific primers when required. Samples were then denatured by heating 5 minutes at 95°C, electrophoresed in a 6% w/v polyacrylamide gel and autoradiographed.

2.2.5 Radiolabelling of Nucleic Acids

2.2.5.1 5′ end labelling of single stranded oligonucleotides with T4 Polynucleotide Kinase

The reaction for 50 ng DNA oligonucleotide was carried out in a total volume of 50 µl. The reaction mix contained 1xKinase buffer (50 mM tris-Cl pH 7.6, 0.1 M $MgCl_2$, 5 mM dithiotreitol, 0.1 mM spermidine, 0.1 mM EDTA), 10 U T4

Polynucleotide Kinase and 20 μCi γ^{32}P-dATP. Samples were incubated at 37°C for 30 minutes before stopping reaction by addition of 2 μl 0.5 M EDTA pH 8.0. Radiolabelled oligonucleotides were purified from unincorporated nucleotides by ethanol precipitation.

2.2.5.2 Random priming of DNA for use as hybridisation probes

Hybridisation probes were generated by random priming 20-100 ng DNA using the "Oligolabelling Kit" supplied by Pharmacia. Reaction were carried out in 50 μl for 30 minutes, 20 μCi α^{32}P-dCTP were used. Radiolabelled DNA probes were purified from unincorporated nucleotides using a NICK column (Pharmacia Biothec).

2.2.6 Enzymatic Modification of DNA

2.2.6.1 Restriction enzymes

Restriction endonucleases recognise and cut within specific sequences of double stranded DNA leaving blunt ends, 5′ and 3′ protruding ends. These were used in the construction and analysis of recombinant plasmids. Each restriction enzyme functions optimally in a buffer of specific ionic strength. All buffers were supplied by the same company that supplied the enzymes and were used according with the manufacturer's instructions.

For analytical digests 100-500 ng DNA were digested in a volume of 10-20 μl containing 5 U of the appropriate restriction enzyme per μg DNA. The digest was incubated for 3-6 hours at the optimal temperature required by the enzyme used.

Preparative digest were made of 1-20 μg DNA using the above conditions but a larger reaction volume. Enzymatic activity was then removed either by incubation at 70°C for 20 minutes or phenol-chloroform extraction.

2.2.6.2 Large fragment of E. Coli Polymerase I

The large fragment of DNA Polymerase I (Klenow) lacks the 5′ to 3′ exonuclease activity of the intact enzyme, but retains the 5′ to 3′ exonuclease activity

and 3′ to 5′ exonuclease activities. It is used to catalyse the polymerisation of deoxyribonucleotide triphosphates in a 5′ to 3′ direction on a template of double stranded DNA with a recessed 3′ hydroxyl and protruding 5′ phosphoryl terminus to generate a flush-ended DNA molecule. This was useful for creating compatible ends for ligation during construction of recombinant plasmids, to digest aspecific A residues added by Taq DNA polymerase at the 5′ terminus and for radiolabelling of DNA fragments by random priming.

Klenow fragment was used with the proper buffer supplied by Biolabs Inc. at a final concentration of 5U per µg DNA. When a "fill-in" was required (DNA fragments with protruding 3′ ends) dNTP for a final concentration of 0.5 mM were added. The mixture was incubate at 37°C for 20 minutes.

2.2.6.3 Dephosphorylation of DNA 5′ termini

Calf intestinal phosphatase catalyses the removal of 5′ terminal phosphate groups from linear DNA molecules. This is used to reduce self ligation of vector DNA during generation of recombinant clones, thus increasing the proportion of resulting recombinants containing the required DNA inserts.

This reaction was carried out in a final volume of 50-100 µl using 1U of enzyme per 0.5 µg DNA incubating for 1 hour at 37°C. The enzyme has then be inactivated by adding 1 mM EDTA and incubating for 20 minutes at 75°C.

2.2.6.4 T4 DNA ligase

T4 DNA ligase catalyses the formation of a phosphodiester bond between adjacent 3′ hydroxyl and 5′ phosphoryl termini in DNA, requiring ATP as a cofactor in this reaction. This enzyme was used to join double stranded DNA fragments with compatible sticky or blunt ends, during generation of recombinant plasmid DNAs.

20 ng of linearised vector were ligated with a 5-10 fold molar excess of insert in a total volume of 20 µl containing 1x ligase buffer and 1 U T4 DNA ligase. Reaction was carried out at room temperature for 6-12 hours.

In some reactions synthetic oligonucleotide were included in the reaction. In these cases amounts added to each reaction to obtain inclusion of oligonucleotides in the resulting plasmid were about 100 fold molar excess over the DNA vector.

2.2.6.5 DNAsi

For the elimination of any DNA genomic contamination after the RNA extraction a DNAsi treatment was performed on the RNA extracted from the tissue.

DNasi I from bovine pancreas is double strand specific endonuclease.

20 µg of total RNA was incubate in a total volume of 100 µl containing 1x DNAsi buffer and 2U DNAsi at 25 C for one hour. The enzyme will be inactivate using a phenol/clorophormio extraction.

2.2.7 Transformation of Bacteria

2.2.7.1 Preparation of competent cells

E. Coli strains were grown overnight in a 200 ml volume of LB at 37°C. Afterwards cells have been incubated in ice for 15 minutes, centrifuged and the pellet resuspended in 20 ml of TPB1 solution (K-acetate 30 mM, KCl 100 mM, $CaCl_2$ 10 mM, $MnCl_2$ 50 mM, glycerol 15%, pH 5.8.) The solution has been incubated in ice for 5 minutes, centrifuged and the pellet resuspended in 2 ml of TPB2 solution (MOPS 10 mM, $CaCl_2$ 75 mM, KCl 10 mM, glycerol 15%, pH 6.5. The solution has incubated in ice for 15 minutes aliquoted and stored at -80°C.

2.2.7.2 Transformation

60 µl of competent cells have been transformed with 1-5 ml of the ligation product and incubated for 30 minutes in ice. The cells have then been incubated for 90 seconds at 42°C. 60 µl of LB have then been added and the cells incubated for 10 minutes at 37°C. The cells have then been plated on agarose plates containing the appropriate antibiotic. When DNA inserts have been cloned in the polylinker of the commercial pUC18 plasmid destroying the β-galactosidase activity of the plasmid 30 µl of IPTG 100 mM and 20 µl of x-Gal 3% have been added on the surface of the plates to test the growing colony for galactosidase activity. The plates have then been incubated for 12-15 hours.

2.2.8 Maintenance and Analysis of Cells in Culture

HeLa, Hep G2, NMuli, 293 cells have been used and all tissue culture work was performed using sterile techniques in a laminar flow tissue culture hood.

The characteristics of the cellular lines are resumed in the appendix A.

2.2.8.1 Maintenance of cells in culture

Cells were propagated in DMEM media and were maintained in 100x20 mm Falcon tissue culture plates, incubated at 37°C and with 5% carbon dioxide.

Plates containing a confluent monolayer of cells were passaged 1 in 5 with trypsin as follows. Cells were washed with 5 ml PBS solution, then were incubated at room temperature with 3 ml PBS/EDTA/trypsin solution (PBS containing 0.02% w/v EDTA and 5% v/v trypsin solution) for 2 minutes or until cells were dislodged. After adding 10 ml DMEM cells were pelleted by centrifugation in polypropylene tubes and were resuspended in 5 ml prewarmed medium. 1 ml of this cell suspension was added to 10 ml medium in fresh plate and was gently mixed before incubation. This procedure was required on average once every three days.

2.2.8.2 Transfection of recombinant DNA into cells maintained in culture

Cells were passage as above into 60x15 mm tissue culture plates and grown to a confluence of 40-80%. The purified recombinant DNA was mixed with lipofectin in the quantity indicate from the manufacture procedure and the mixture was incubated for 15 min to allow the formation of DNA-liposome complexes. The mixture was added to the cells in 2 ml of serum free DMEM and incubated at 37 C for up to 24 hours.

2.2.8.3 Vaccinia virus infection

6-8 hrs before transfection cells were washed and fed with fresh medium (DMEM + 10% FCS) . 30-40 min before infection the virus was thawed . Meanwhile, the cells were washed twice with PBS and when the incubation is over, a cocktail of virus and serum free DMEN was added to the cells. Virus was normally used at moi

of 30 pfu and incubated at 37 C for 30 min. After 30 min of adsorption, the inoculum was aspirated and proceeded with transfection with lipofectin as described above.

2.2.8.4 Analysis of the Cre recombinase activity and HCV Core protein presence into cells maintained culture

The purified plasmid Bls906Corelox/Lac-Z and pΔE1 sp1A were cotransfected in Hep G2 and NMuli cells with lipofectin. After 24 or 48 hours depending on the utilisation or not of the Vaccinia virus infectious system, the medium was collected and the cells were scraped off in PBS and aliquotated. The cells were pellet and the supernatant was aspirated. These cell pellets were frozen at-80 C and were used later for determination of Cre recombination activity, after previous sonication, using western blot analysis.

SDS-Page was performed according to the method of Laemli. Cellular extract expressing HCV Core protein were resuspended in denaturing sample buffer containing 4% SDS and 3% dithiohreitol. After 5 min incubation at 100% the samples were loaded on to 15% polyacrilamide gels and subject to electrophoresis. Western blotting of the proteins to PVDF membrane was performed for 2 hrs at 200 mA. After transfer the membrane was stained with Ponceau Red S, washed and blocked for 30 min in PBE containing 5% skimmed milk. The expected product of the Cre mediated recombination was detected by incubation with anti-Core monoclonal antibody (B12.F8 a generous gift of Dr Mondelli) followed by an HRP conjugated anti human IgG.

2.2.9. mRNA Analysis by polimerase chain Reaction

2.2.9.1 Amplification of RNA by reverse transcription and polymerase-chain reaction

To detect the transgene RNA transcript , 2μg of total RNA from tissues was mixed with 17 μl of buffer containing 10 mM Tris-HCL (pH 8.4), 50mM KCL, 2.5 mM $MgCl_2$,1mM of each dNTPs (Pharmacia, Uppsala, Sweden).The appropriate specific primers(as indicated in table 2.1) was used in each reaction. After addition of

0.1U/µl of RNasin (Boeringher Mannheim, Mannheim Germany), and 10 U Moloney murine leukaemia virus reverse transcriptase (MMLV-RT,BRL,Milan,Italy), the reaction was incubated at 37 C for one hour.

The polymerase chain reaction was performed following the basic protocols of the Boehringer and Promega Taq DNA Polymerase. The volume of the reaction was 50-100 µl with 1x Taq buffer, dNTP mix 200 µM each, oligonucleotide primers 2 µM each, Taq DNA Polymerase 2.5-5 U. As template were used 1.5-3 µl of the cDNA reaction mix.

The amplifications were performed on a Perkin Elmer Cetus DNA Thermo Cycler.

PCR reactions were performed in a final volume of 100 µl. Each cycle was carried out as follows: denaturation step (1 minute at 93°C), annealing step (1 minute at 56°C), extension step (1 minute, 30 seconds at 72°C). The number of amplification cycles ranged from 25 to 30. Primers sequence are shown in the table 2.1

Control reactions for cDNA synthesis and following PCR amplification were set up including all the reagents except the RNA sample and were routinely performed for each series of RT-PCR assay.

Is possible to increment the sensibility of the amplification with second round of PCR . For this experiment 3 µl of the first PCR reaction mixture was amplified using other specific primers localised in an internal region respect to the primers used in the first PCR amplification.

2.2.9.1 3′ Race

The same approach described previously was used to perform a 3′ Race experiment. Also in this case 2µg of total RNA was reversed transcribed and the same quantity of cDNA was used to perform the PCR amplification.

Specific primers were used in this experiment. The sequence of the primers is summarised in the table 2.1 . Each cycle was carried out as follows: denaturation step (1 minute at 94°C), annealing step (1 minute at 55°C), extension step (3 minute,at 72°C). The time of elongation ranged according with the aspected amplified size.

2.2.9.2 PCR amplification performed on the genomic DNA Expand Long Range PCR

In the cases of genomic DNA amplification a Expand Long Template PCR system was used according to the manufactures instruction.

PCR reactions were performed in a final volume of 100 µl adding 100-200 ng of DNA. Each cycle was carried out as follows: denaturation step (1 minute at 93°C), annealing step (1 minute at 68°C), extension step (4 minute, 30 seconds at 68°C). The number of amplification cycles ranged from 30 to 35

The sequence of the utilised primers is reported in the table 2.1.

2.2.10 Recombinant Ad virus

The defective recombinant Adenovirus was prepared according with the protocol proposed by Graham L (Graham and Prevec, 1991).

2.2.10.1 Preparation of plasmid pΔE1sp1A and adenovirus genome DNA pJM17

The recombinant shuttle vector pΔE1sp1A was transformed to the competent cells DH5α. The entire adenovirus genome DNA pJM17 was transformed using the competent cells HB101 or LE392 that present a higher efficiency of transformation. Transformed bacterial growth very slow and the colonies are very small. The transformed bacterials are not suitable for longer storage.

2.2.10.2 Amplification of bacterial and preparation of genomic plasmids pJM 17

The plasmid-bearing bacteria was streaked on a LB-agar plate containing appropriate antibiotics, and grow overnight at 37 C

The day after two or more colonies were picked off the plate and resuspended in 5 ml LB plus antibiotics and incubate at 37 C on a shaker for several hours. Each 5 ml culture were added to 500 ml LB plus antibiotics and continue incubation overnight.

The DNA plasmid was purified by alkaline lysis of the bacterial and CSCl banding as described in standard cloning manual (Sanbrook, 1989).

2.2.10.3 DNA transfection for rescue of recombinant Adenovirus vectors: calcium phosphate coprecipitation

For an efficient cotransfection of the two plasmid pJM17 and pΔE1sp1A the shuttle vector was linearised. 20μg of DNA purified by CsCl gradient centrifuge was digested using the appropriate restriction enzyme for two hours at 37 C. If the digestion was complete after this time the liner DNA was purified using a Phenol/Chloroform extraction

Purified adenovirus genome DNA pJM17 was directly used in cotransfection

Monolayer culture of 293 cells are grown in P60 dish at 50% confluence. The medium (DMEM plus 10% FCS) was change 1 hour before transfection.

The cotransfection was performed utilising the Calcium-phosphate DNA precipitation according with the classical protocol reported in the Maniatis cloning manual (Sanbrook, 1989). 10 μg of pJM17 and 15μg of linearised recombinant DNA was normally used.

After cotransfection the cells were incubated for 16-18 hours under standard growth conditions. After this time the medium was removed and replace with 5 ml of complete DMEM medium. The cells was leave for 7 days without changing medium.

After this time the cell were freeze and thaw 3 time, centrifuge at 2500 rpm 5 min a RT. 3 ml of the surnatant was used for infect a fresh dish of 293 cells at 50% on confluence. The infected cells were incubated at 37 C for 3 hours, the surnatant was aspirated and stored at -80C. The medium was replace with a fresh DMEN plus 5% FCS.The cells were incubated for 6-7 days in standard growth condition and then the same procedure was repeat. If recombination take place , clear cytopathic effect is seen in the 3rd or 4th passage.

2.2.10.4 Limited dilution and identification of the recombinant Adenovirus.

The supernatant of the passage in which was visible a chitopathic affect was diluted with DMEM without FCS from 10^{-1} to 10^{-7} and the dilution was performed directly in 96 wells plate. Approximately 10000 fresh 293 cells were seed in every wells. The plate was incubated for 3-5 days in normal growth condition. After this time the surnatant was removed to a new 96 well plate that was conserved at 4C and lysed cells was used for Dot Blot hybridisation.

2.2.10.5 Dot blot hybridisation for the identification of the recombinant Adenovirus

The cells were lysed with 0.5 N NaOH 200 µl well at room temperature for 5 min. Nh4 Ac 10 M was added for neutralisation. To each well were added 20 µl of neutralising solution. The sample was fixed on a Nylon membrane using a dot blot apparatus connects with vacuum. The membrane was washed with 0.02 N NaOH-1M Nh4Ac directly with 100 µl in each wells. Remove the membrane from the apparatus when solution was passed throughout the membrane and dry it for 10 min a room temperature. Fix DNA on the membrane using cross linking.

The membrane was incubate at 65 C with 0.25% milk-5X SET for 1 hour with shakes. The hybridisation was performed adding the probe directly to this solution and the temperature of hybridisation was chosen depending in the size of the probe. (65 C for a DNA fragment, 42 C for a oligo).

Non-specific hybridisation of probes to the filters was reduced by multiple washes at decreasing salt concentrations as required. The following protocol was used:

a)30 min 2X SET-0.5% SDS

b)30min 1X SET-0.2% SDS

c)30min 0.2X SET-0.2% SDS

The filter was then dried and exposed using X-O-Mat "S" film (Kodak) at room temperature or at -80°C with an enhancer screen for the required time.

The clones characterised by a more strong radioactivity at higher dilution were amplified by infection of fresh 293 cells.

2.2.11 Analysis of β–galactoidase activity

For detection of β-gal activity in cell colture or in mice tissues, the cells or the tissues were fixed in 2% gluteraldehyde solution for 5 min at 4 C, washed with PBS three time for 10 min and later incubated in stain solution(8.4 mM KCl, 1mM MgCl2, 3mM $K_4Fe(CN)6$, 3mM $K_3Fe(CN)6$, 3% 5-bromo.4-clore-3-indolyl β-D-galactopyranoside (X-Gal)) overnight at 37 C.

2.2.12 Analysis of HCV Core protein expression

2.2.12.1 Western blot analysis

Livers of transgenic mice and nontransgenic littermates were homogenised in a buffer containing 100mM/L Tris-HCl pH 6.8, 2.5% SDS , 6%s sucrose and a cocktail of protease hinibitors.Protein concentration was determined using the Bio-Rad protein assay method (Bio-Rad).

50μg of total protein were loaded on a 15% sodium dodecil sulphate-polyacrilamide gel electrophoresis, and transferred onto a supported membrane(nitrocellulose extra blotting membrane Sartorius) by electronblotting as described previously in the 2.2.8.4 section of this chapter.

The membrane was blocked in PBS containing 5% non fat dray milk for two hours at room temperature. The primary antibody B12.F8 was added at a 1/100 dilution in PBS containing 2%non fat dray milk. After incubation with the primary antibody, the membrane was washed extensively in PBS. The appropriate horse radish peroxidase conjugate second antibody was added a 1:1000 dilution in PBS ,2% non fat dray milk for one hours. The membrane was washed with 3 changes of PBS

and the detection of the protein was performed with an Henanced Chemioluminescence Detection kit (Amersham).

2.2.12.2 Immunohistochemistry

Livers from 1,6 and 16 weeks were fixed in 4% parafolmaldehyde in PBS,sectionated and subjected to himmunohistochemistry using a Vectastain ABC kit (Vector Laboratories,Burlingame, CA). Anti HCV Core monoclonal antibody (B12.F8), rabbit polyclonal antibody and anti HCV Core human sera were used as primary antibodies at concentration ranging from 1/100 to 1/10 in PBS. The slides were incubated for at least one hour in a humidified chamber at room temperature.After washing with PBS ,secondary antibody (biotin conjugated rabbit anti human or anti mouse) was applied at a 1/100 dilution for 30 min . as negative control, non immune rabbit serum or BSA were used.

The slides were counter stained with hematoxilin.

2.2.13 ELISA procedure

Protein and synthetic peptide (0.2µg/well) in 200mM carbonate buffer (pH 9.6) were bound to a 96-well microplate (MaxiSorp;Nunc) by incubation them in a microplate overnight at 4 C. The peptides and proteins that were absorbed were washed three times with TTBS(50mM pH: 7.5) NaCl (500mM) and 0.1% tween 20) and were incubate with the transgenic mice sera at room temperature for 1 hour. After three further washes with TTBS the colour was developed with ortho-phenylendiamine dihydrochloride (Sigma-Fast;Sigma Chemical Co,St. louis, Mo). The reaction was stopped after 5 min by adding 3 M sulphuric acid and the optical density (OD) was measured at 492 nm (OD 492). All data points are the means of triplicate determinations.

PRIMERS	SEQUENCE	USE
Transf-X (antisense)	CTGATCCTCGAGCTT AAGTAGGTGTGTGTG CGAGGATGG	Amplification trans. promoter
Transf-K/N (sense)	CTCGGAGGTACCTCG CGAGTAATACGACTC ACTATAGGGCCTTAA GCCAGAGCAGGCCAG GCCT	Amplification trans. promoter
5′ UTR-X (sense)	CCAGGCCTCGAGTGT GAGGAACTACTG	Amplification 5′ UTR
5′ UTR-H-lox (antisense)	CGTATCAAGCTTATA ACTTCGTATAGCATA CATTATACGAAGTTA TCGTGCTCATTGGTG CACGGTCTAC	Amplification 5′ UTR
β-gal-H (sense)	ATCCGGAAGCTTCCA TGAGCGAAAAATACA	Amplification β-gal
β-gal-B-stop (antisense)	CGGACTGGATCCTCA CTATTATTTTTGACAC CAGAC	Amplification β-gal
Core-B-lox(sense)	CGTACAGGATCCATA ACTTCGTATAATGTA TGCTATACGAAGTTA TCCAATCTTAAACCT CAA	Amplification HCV Core
Core-X-906 (antisense)	CAGATTTCTAGATCA TTAAAGCGGAAGCTG GGGTGG	Amplification HCV Core (192 aa)
Core-X-700 (antisense)	CAGATCTCTAGAATC GATGACCTTACCCAA ATT	Amplifiaction HCV Core (ClaI)
Core 718 (antisense)	TCAGGCAGGACAGC AGAGCCAAG	cDNA core synthesis
Core 800 (antisense) amplification	GAACCCGGACACCA TGTGCCAG	Transgene

Core 906 (antisense) amplification	AGCCGCATGTGAGGG TATCGATGACCTTACC CA	Transgene (Nested PCR)
Lac-Z 1502 (sense) amplification	TCAGCCGCTACAGTC AACAGCA	Transgene
Lac-Z 1380 (sense)	TCAGTATCGGCGGAA TTCCAG	Transgene amplfication (Nested PCR)
Lac-Z 2584 (sense) amplifiaction	CAGTTCTGTATGAAC GGTCT	Transgene
Core 790 long range AS	ACGCCGTCCTCCAGA ACCCGGACACCATGT GCCAG	Recombinant and non non recombinant fragm. amplifiaction
5′ UTR 4720 long range S	AGACTGCTAGCCGAG TAGTGTTGGGTCGCGA AAGG	Recombinant and non non recombinant fragm. amplifiaction
5′ UTR 4710 (sense)	AGACTGCTAGCCGAGT TAGTGT	cDNA amplification
RACE 3′dTIB (antisense)	AACTGGAAGAATTCGC GGCCGCAGGAAT(18)	Reverse transcriptase
RACE anchor (antisense)	AACTGGAAGAATTCGC GGCCGCAGGAA	cDNA amplification
RACE 799(sense)	AGGACGGCGTGAACTA TGCAACAGG	cDNA amplification
RACE 896 (sense)	TCACATGCGGCTTCGCC GATCTCAT	cDNA amplification
GC 106 (antisense)	AGCGGCTGATGTTGAAC TG	β-gal amplifiaction
GC 105 (sense)	TTGGCGTAAGTGAAGCG AC	β-gal amplifiaction

Cre rec dir 1 (antisense)	CGTCTAGATCGCGAGATA TCCATGTCCAATTTACTGA CCGTAC	pΔE1sp1a Cre/NLS amplifiaction
Cre 775 (sense)	ATCCGCTCGACCAGTTTA GTTAC	pΔE1sp1a Cre/NLS amplifiaction
Cre 162 (S14) (sense)	AACGAGTGATGAGGTTCG	Cre gene probe
ForB-actinEX(sense)	CATGTTTGAGACCTTCAA CA	β–actin amplification
RevB-actin EX(antisense)	ATCTCCTTCTGCATCCTG TC	β–actin amplification
5′ GAPDH (sense)	ACATGTTCCAGTATGAC TCT	GAPDH amplifiaction
3′ GAPDH (antisense)	ACGGAAGGCCATGCCAG TGA	GAPDH amplifiaction

Table 2.1: sequence and employment of the oligonucleotide primers used in this research.

APPENDIX A

Name: Hela

Tissue: Epithelioid carcinoma, cervix.

Species: human

Virus susceptibility: susceptible to poliovirus type 1 and adenovirus type 3

Morphology: Epithelial-like

Growth: Monolayer

Medium: minimum essential medium (DMEM) with 10% FCS

Comment: HeLa was the first aneuploid, epithelial-like cell line to be derived from human tissue and mantained continuously by serial cell colture

Name: 293

Tissue: kidney;transformed with adenovirus 5 DNA

Species: human; embryo

Virus suspect: very sensitive to human adenoviruses

Tumorigenic: yes in nude mice

Morphology: ephithelial

Growth: monolayer

Medium: Dulbecco's modified eagle's medium with 1 gr/L glucosse 90%, fetal bovine serum 10%

Fluid renewal: every 2 days

Comments: the cells express the E1 transforming gene of adenovirus 5;adenovirus 5 DNA from both the right and the left ends of the viral genome are present the line is excellent for titration human adenovirus

Name: NMuli

Tissue: liver

Species: mouse; weanling

Virus suspect:no

Tumorigenic: yws,cystadenomas with malignant characteristics are produces in isogenic mice

Morphology: ephithelial

Growth: monolayer

Medium: Dulbecco's modified eagle's medium with 4.5 gr/L glucosse 90%, fetal bovine serum 10%

Fluid Renewasl: 2 to 3 times weekly

Name: Hep G2

Tissue: hepatoblastoma; liver

Species: human

Virus suspect:no

Tumorigenic: no

Morphology: ephithelial

Growth: monolayer

Medium: Dulbecco's modified eagle's medium with 1 gr/L glucosse 90%, fetal bovine serum 10%

Fluid Renewasl: 2 times weekly

Comments: there is no evidence of hepatitis B genome in this cell line. The cells express 3-hydroxy-3-methylglutaryl-CoA reductase and hepatic triglyceride lipase activities. It is not an hybridoma

Chapter 3

Results

3.1 TRANSGENE PRODUCTION STRATEGY

The analysis in isolation of an autoimmune pathogenic mechanism provoked by HCV gene product required the absolute certainty that the HCV proteins were not expressed even minimally in the transgenic mice until triggered by an external factor . From this event on wards the expression should be constitutive and for as long period as possible. To obtain this , it was decided to design two different types of vectors in allowed the expression of either the Core protein alone or of all the viral proteins codified by the HCV genome only after a recombination events. The introduction of the Cre recombinase protein in a transient manner, through the development of an Adenovirus vector, will then be used to cause the rearrangement between the two lox P sequences, the elimination of the Lac-Z gene in a circular form, and the expression of the HCV proteins in a controlled mechanisms . The overall strategy is shown in Fig.3.1.1. Also if both vectors share the same characteristics (Fig 3.1.2) and the same assembly mechanisms (Fig.3.1.3 and Fig.3.1.4) initially, our efforts focused on obtaining the vector **Bls 906 Core loxP/Lac-Z** with the aim of expressing the entire HCV Core protein (residues 1-192). This interest was motivated by the reason that the Core protein presents a lot of characteristics that associate its presence with the progression of the hepatitis C virus infection and possibly with several other pathologies associated with this disease, such as autoimmunity. Moreover, it has been observed that most of the immunological response against the hepatitis C virus is directed against this protein.

This Bls906Corelox/Lac- Z construct has been obtained following a strategy of sequential assembly of different products of PCR amplifications which is summarised in Fig 3.1.3. Different sets of primers were used for each amplification

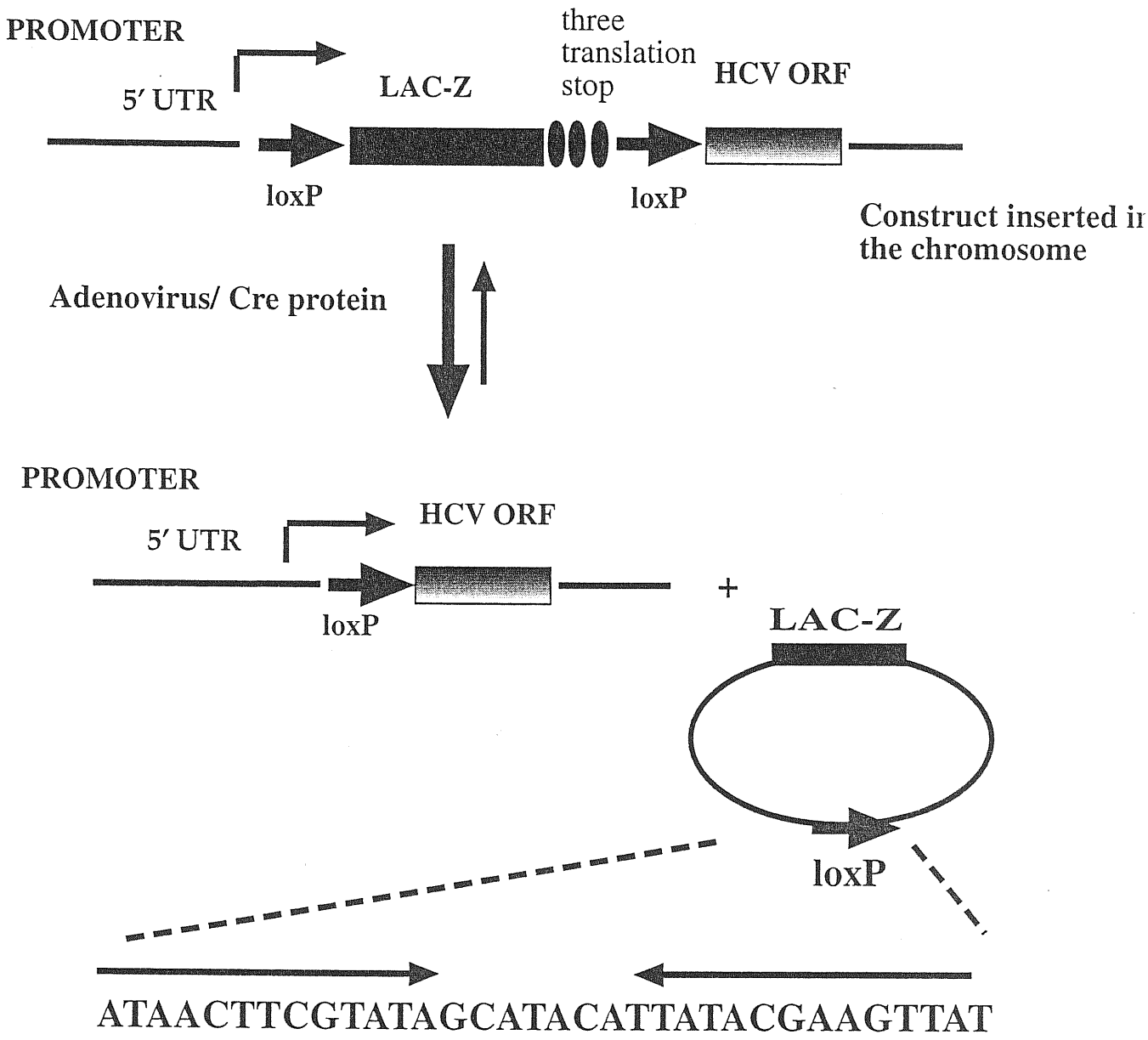


Fig 3.1.1: The Adeno/Cre mediated recombination between the two loxP sequences allow the excision of the Lac-Z gene and the switching on of the HCV Core protein.

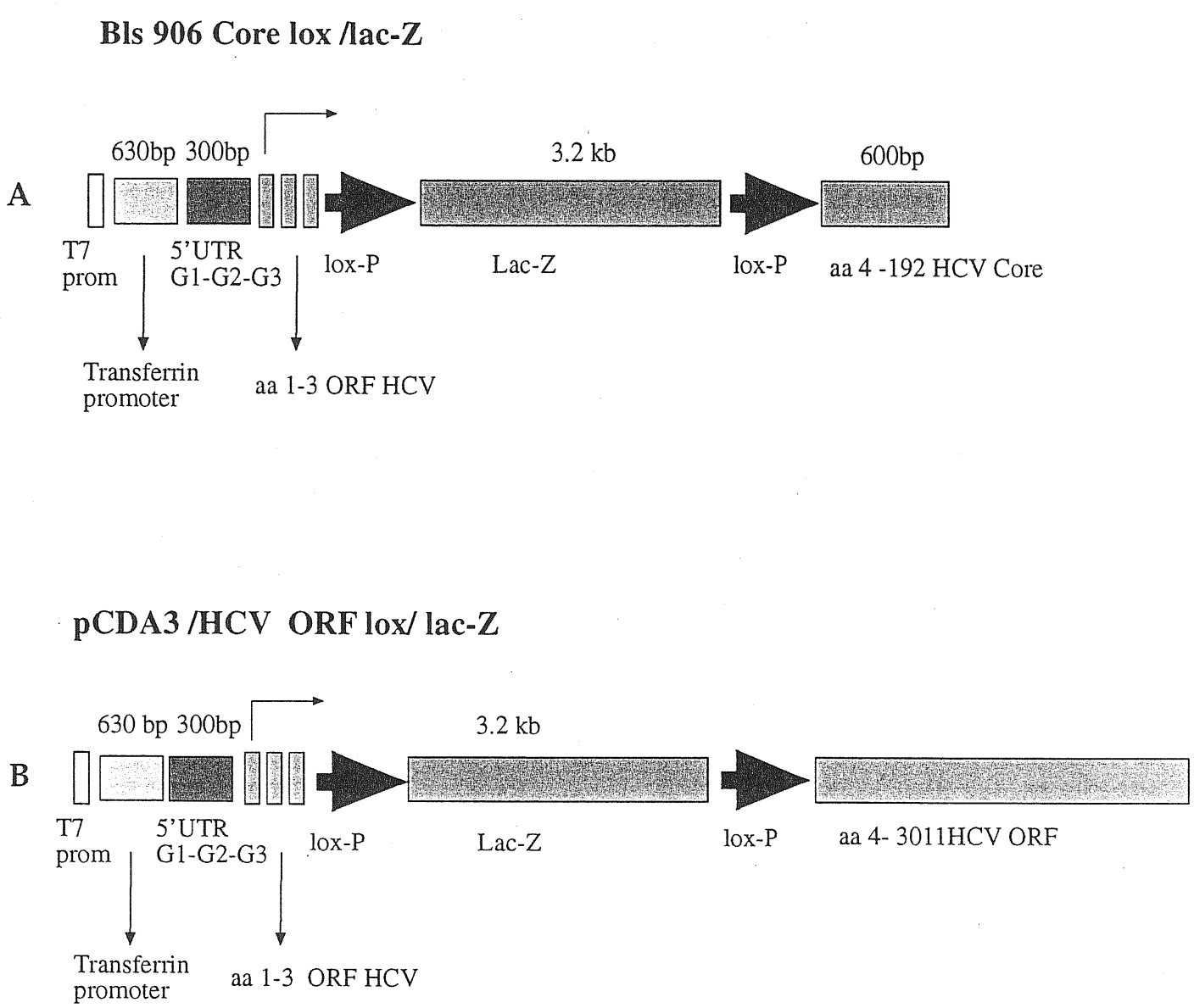


Fig 3.1.2 : Final organisation of the two constructs designed to obtain the expression of the HCV Core protein alone (panel A) and of all the proteins of the HCV virus (panel B) after the Cre mediated recombination. Both constructs contain a tissue specific fragment(-581 +50) of the transferrin promoter, the 5'UTR of the HCV virus, the two loxP sequences and finally the Lac-Z gene introduced as stuffer DNA .

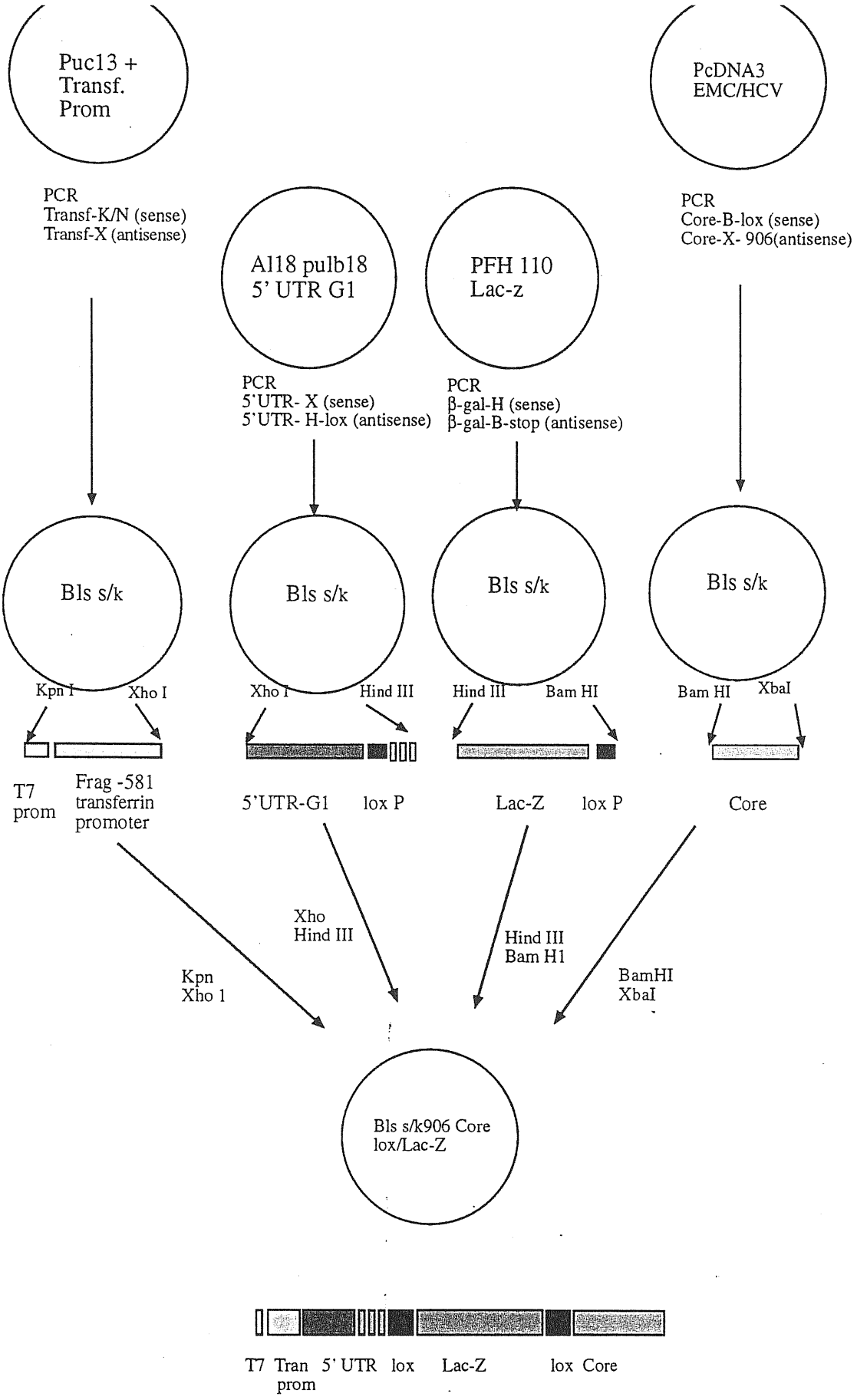


Fig 3.1.3: Strategy used to obtain the vector Bls 906Corelox/Lac-Z

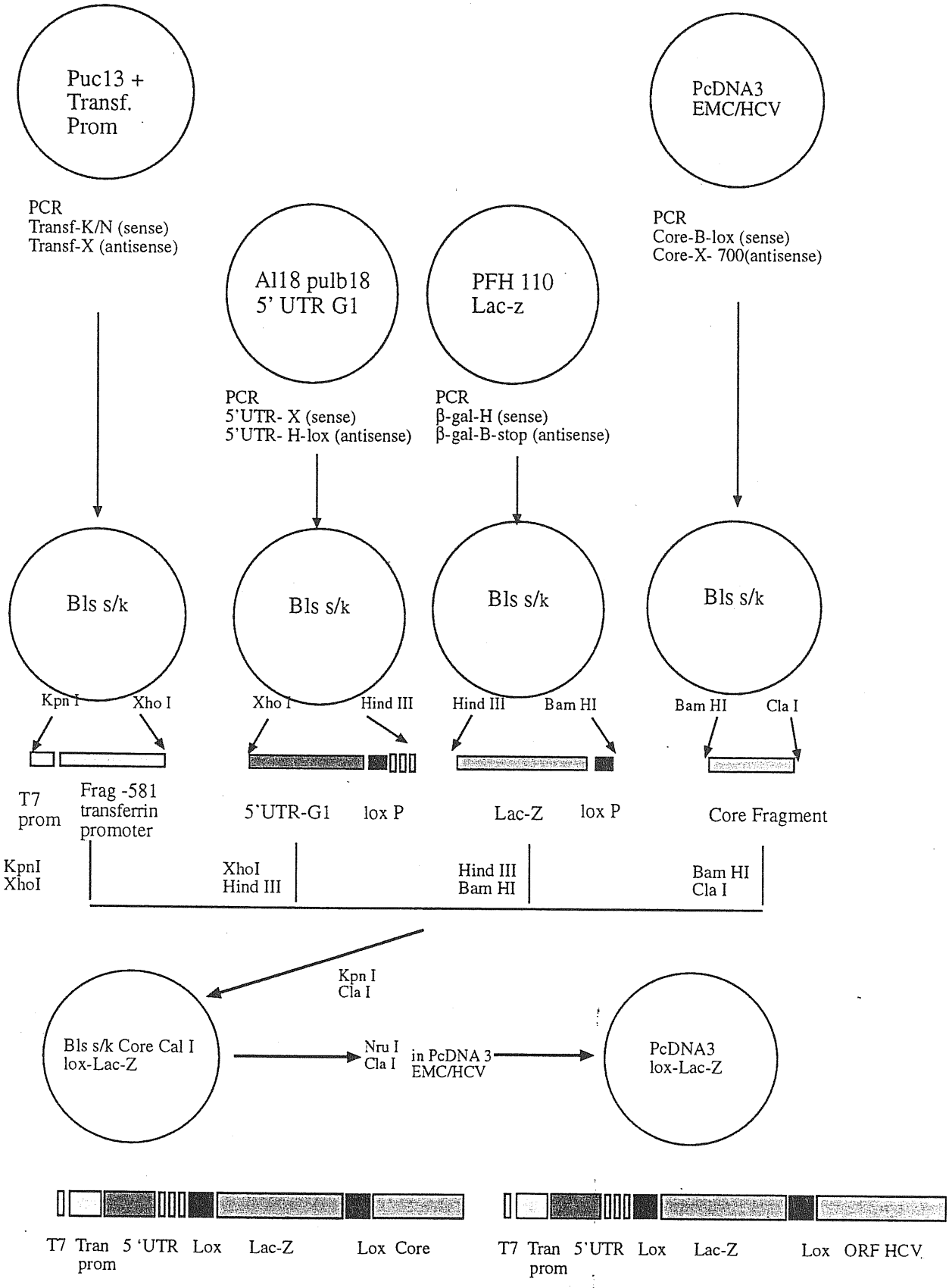


Fig 3.1.4: Strategy used to obtain the vector pcDNA3/HCV ORF lox/Lac-Z

and the sequence of all the primers used in this work is summarised in the table 2.1 reported in the Material and Methods section .

Each primer was designed to ensure the maintenance in frame of the nucleotide sequence of the HCV Core protein following the recombination event. The same method was used to engineer in the C-terminal region of the Lac-Z gene three translation stop codons to cover all three reading frames. These stops prevent also the minimal expression of a leaky HCV Core protein before the introduction of the Cre protein.

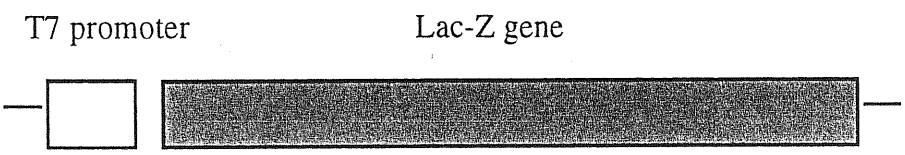
The amplified fragments were joined together by the introduction of particular restriction enzyme sites which were inserted in the each of their sequences. The Transferrin Promoter fragment, the 5′ UTR and the Core (respectively 631, 341 and 600 bp long) were entirely sequenced to exclude a possibility of PCR-introduced mutations.

On the other hand, since the total sequence of the entire 3.2 kb Lac-Z gene product would have been too time consuming, we tested functionally the amplified β-gal protein by performing a transfection assay. This experiment was based on the staining of Hela cells transfected with the amplified Lac-Z fragment cloned in the Bls vector and placed under the control of the T7 promoter (as described in Material and Methods). In order to increase the efficiency of the β-Gal protein production during the transfection we used a Vaccinia virus system as described in Material and Methods (Fig 3.1.5). The results showed that this amplified product is capable of coding for a functional β-gal protein.

3.2 CHARACTERISTICS OF EACH SEGMENT IN THE FINAL CONSTRUCT

3.2.1 The promoter : A fragment of the transferrin promoter

The transferrin protein is an essential iron binding protein that is responsible for transporting iron throughout the body (Morgan, 1983). The major site of



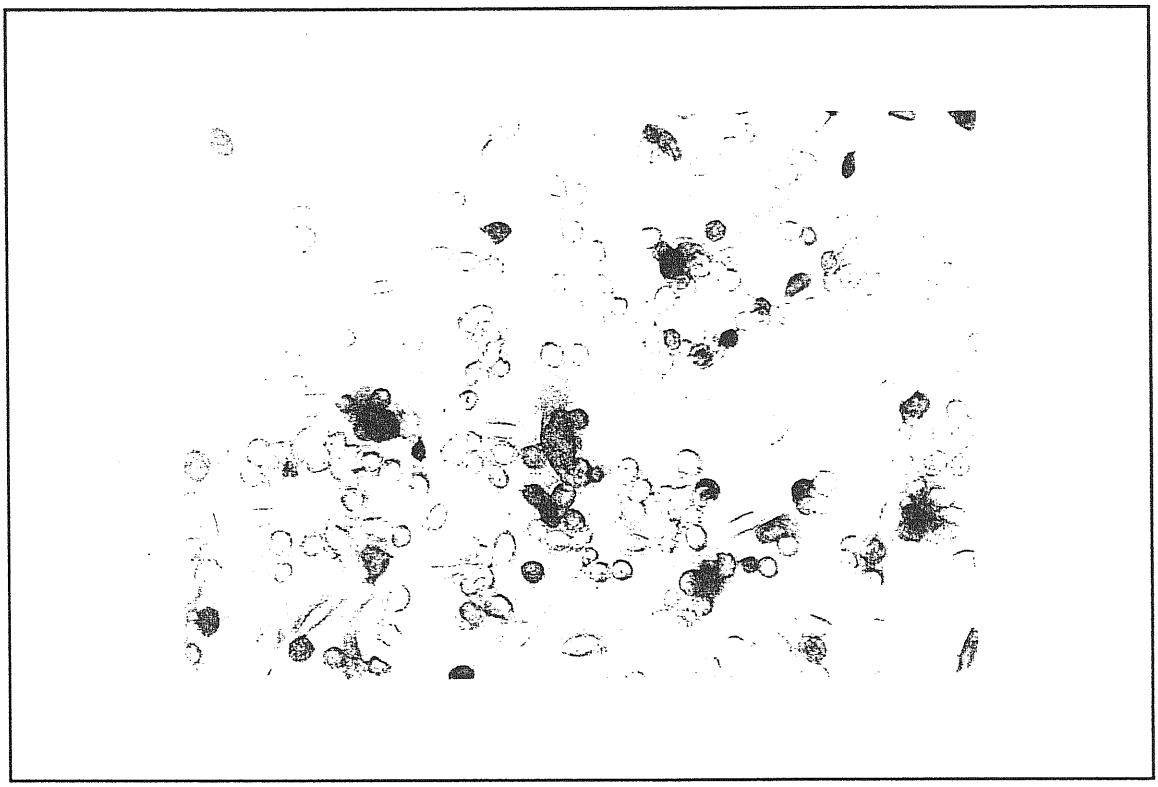


Fig 3.1.5: Before insertion of the amplified Lac-Z product in the Bls 906Corelox/Lac-z vector, β-gal protein viability was validated by performing a functional transfection assay based on staining of Hela cells transfected with the amplified Lac-Z gene cloned in Bls vector under the T7 promoter.

transferrin synthesis is the liver (although other tissues produce transferrin in significant amount). We have focused our attention on this gene since it contains a promoter capable of directing expression of a reporter gene in several highly differentiated tissues in the chicken (Cochet et al., 1979),human (Lucero et al., 1986) and mouse ((Idzerda et al., 1989).

In particular, several studies had already been performed with the aim of understanding which regions of human transferrin promoter was responsible for liver-specific expression of the transferrin gene. Transfection experiments performed on human cell lines and in transgenic mice demonstrated that the proximal promoter region of the human Tf gene is not sufficient for tissue specific expression in these two experimental systems (Adrian et al., 1990). However, a previous work by Schaeffer et all (Schaeffer et al., 1989) speculates that the proximal elements of the promoter are sufficient for constitutive tissue-specific expression of the Tf gene in the liver.

A different situation has been observed regarding the mouse transferrin promoter. Recent experiments using a transgenic animal system have allowed to compare the effects of the mouse transferrin promoter with its human homologue (Bowman et al., 1995). The results allowed to clarify which regions of the entire 3kb transferrin promoter are necessary to drive the tissue-specific expression of the reporter gene. In particular, this allowed to isolate the minimum region capable of driving the expression of the reporter gene above all in the liver, although good levels of expression were also observed in other tissues such as the brain, kidney and heart. To do this fusion genes containing either 3Kb, 581 bp and 139 bp of upstream murine Transferrin genomic sequence linked to the hGH structural gene region were used to produce transgenic mice. All these animals expressed substantial levels of growth hormone mRNA in the liver (Idzerda et al., 1989).

The fact that a fragment of 139 bp upstream the transcription start site (+50) caused a liver-specific expression of the reporter gene confirmed the hypothesis that

the major liver specific element is located very close to the transferrin coding sequence.

In keeping with this, Theisen et all (Theisen et al., 1993) have observed that a region between nt -98 and -83 of the transferrin gene is very important for liver specific expression of the protein. This region contain the CCAAT consensus sequence that binds to members of the C/EBP (CAAT enhancer-binding protein) family of transcription factors. These results are in agreement with those reported by (Brunel et al., 1988) for the human transferrin promoter. It is also interesting to note that the C/EBP transcription factor has been identified as a key control element of many liver specific genes (Metzger et al., 1990). In addition another consensus sequence for a second liver enriched transcription factor , Tf-LF1(HNF-4) is localised in human downstream of the C/EBP site (Schaeffer et al., 1989; Schmits and Galas, 1979)and since mouse Transferrin gene is highly homologous in this promoter-proximal region, Theisen and co-worker have hypotised that this two transcription factors are likely to be essential for liver specific expression also in the mouse. Studies which used point mutations of these consensus sequence show that the presence of this region is extremely important for liver specific expression but is not essential for the expression in other tissue such as the brain.

In additon,several evidences suggested that the sequences between -139 and -581 bp doesn't substantially change the promoter tissue specificity but increases the level of expression in the liver about fivefold in comparison with the shorter promoter. This fact probably reflects the presence of an enhancer like element in this distal promoter region. Therefore, a co-operative action of this kind of enhancer element with the sequences found in the minimal region of the trasferrin promoter make this region a good candidate to drive a high tissue specific expression in the liver. Therefore, in agreement with this work we decided to include the -581 +50 bp fragment of the murine Transferrin promoter in our transgenic mice. In fact, this fragment has been shown to maintain a level of expression only slightly lower than the entire 3 Kb promoter and, in addition, it showed a good liver specificity .

3.2.2 The 5′ UTR region

The 5′ UTR of the HCV virus is the region able to promote the translational initiation of its genome. In a recent work Buratti et al (Buratti et al., 1997), using a cell culture system, have suggested that there are differences in the translation initiation ability of the 5′ UTR belonging to genotypes 1, 2 and 3. In particular, genotype 3 was the least efficient to promote translation initiation, whilst genotypes 1 and 2 present more or less the some level of efficiently.

Therefore, we were also interested in developing a system with the possibility of replacing the 5′ UTR region with that of different genotypes. Fig. 3.1.2 shows that the strategy used in constructing our vectors allows such a change very easily.

3.2.3 The Cre-lox system

The most essential feature of our work was the possibility to switch on the expression of the HCV protein in a controlled manner. This is made possible by the use of a recombination system that induces precise rearrangements of DNA without any requirement for extensive homology.

One of the simplest site-specific recombination system that could be chosen to achieve this aim, in terms of the relative complexity of its enzymology and recognition sequence, is derived from the lysogenic bacteriophage P1. This phage encodes for a 38-KDa protein (Cre) that has the ability to recombine DNA between specific 34 bp sequences, termed loxP sites (Sternberg and Hamilton, 1981;Sternberg and Hamilton, 1981). This Cre recombinase was also known to be efficacious in mediating recombination in mammalian cells (Fukushige and Sauer, 1992; Lakso et al., 1992; Orban et al., 1992; Sauer and Henderson, 1988; Zhang et al., 1996)

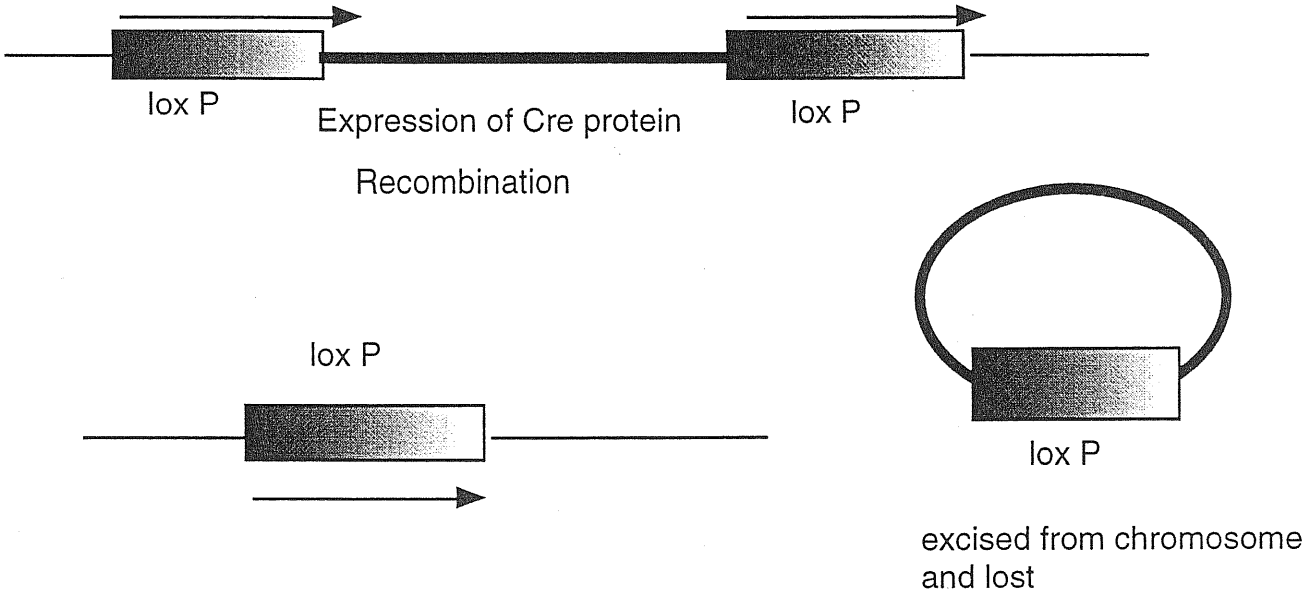
Each loxP site contains a dyad of 13 bp separated by an 8-bp spacer that gives a 5′ to 3′ orientation to the motif. The recombination is initiated by Cre proteins binding to the 13 bp inverted repeat region in the loxP sites (Hoess and Abremski, 1985) with a subsequent strand exange between the asymmetric 8-bp central spacer sequences by concerted cleavage and rejoining reaction, which involve a transient

DNA-protein covalent linkage and which is strictly restricted to these identical sequences.

It is interesting to note that the recombination mediated by the Cre-loxP system is independent from the presence of high energy co-factors or accessory proteins, and is not dependent by the state of super coiling of the DNA substrate or any other topological feature (Abremski and Hoess, 1984; Abremski et al., 1983; Hoess and Abremski, 1984). The Cre protein is capable to catalyse three forms of recombination: excision, inversion and integration, and which form takes place is dictated by the orientation of the loxP sites relative to each other. Excision or inversion occurs when the loxP sites exist on the same strand of DNA. In particular, excision occurs when the two lox sequences are oriented in a parallel manner whereas inversion occurs when the orientation is anti parallel. Finally, insertion can occur by using loxP sites on separate strands.

All these possibilities have made this system particularly suitable for the controlled manipulation of the DNA in cultured mammalian cells (Sauer and Henderson, 1989) and in transgenic mice (Gu et al., 1994; Lakso et al., 1992). Thus, Cre-mediated recombination can be applied to various types of gene manipulation techniques including the removal of a selectable marker gene accompanied by the introduction of subtle mutation (Gu et al., 1993), gene replacement (Zou et al., 1993) and the deletion of large genomic regions to inactivate genes or gene clusters.

The following table summarises the recombination mechanisms that occur during Cre-lox recombination when the two loxP sequences are present on the same chromosome in a parallel orientation .



As previously described, in our construct the two loxP sequences are arranged in a parallel manner and localised upstream and downstream of the stuffer Lac-Z gene sequence. This stuffer DNA causes the disruption of the promoter/coding region structure required for expression through its excision deletion. In this system, the presence also in a transient manner of the Cre recombinase will allow the excision of the Lac-Z gene and the expression of the HCV Core protein.

3.2.4 The Lac-Z gene

The amount of DNA that can be placed between the two loxP sequences in the Cre recombination system can be very large (some kb). In our system the entire coding region of the Lac-Z gene was chosen as stuffer DNA between the two loxP sequences. In the absence of the Cre protein the Lac-Z gene prevents read through expression of the Core protein. Conversely, when the Lac-Z gene is excised by Cre-mediated recombination between the tandem loxP sites the expression of the Core protein is then made possible.

Moreover, at the end of the Lac-Z gene sequence we engineered three translation stop codons to cover all the three frame. The presence of these stop sequences exclude the risk of a possible, also if unlikely, leaky expression of residues

4-192 of the Core protein also in the absence the Cre protein. Such a possibility had to be eliminated completely since any leaky expression of the Core protein would have also eliminated the possibility to present this protein as a "non self " protein to the immunological system of the mice.

3.2.5 The Core protein

We have placed the first three aminoacid of the Core protein at the 3' end of the 5'UTR (Fig.3.1.2), an arrangement that allows to maintain the ATG of the Core protein immediately downstream the 5' UTR. This arrangement mimics the original viral situation in which the Core protein sequence is always located at the 3' end of the 5' UTR. The rest of the entire ORF (4-192) is localised downstream of the Lac-Z gene. It is important to note that when the Cre-driven recombination occurs the elimination of the stuffer Lac-Z DNA creates a full Core protein which contains a insertion of 34 bp between residues 3 to 4 owing to the fact that one loxP sequence will remain. The residues introduced by this insertion (KTSNVCYTKLS) were observed not to affect the immunological response to the protein, the only effect being that of generating a Core protein slightly larger than the wild type.

3.3 FUNCTIONALITY OF THE BLS 906 CORE LOX/LAC-Z CONSTRUCT IN A CELLULAR SYSTEM

Before using the Bls 906 Corelox/Lac-Z construct in the production of transgenic animals several preliminary tests were performed to check for correct functionality in an in vitro system.

First of all we used two different types of experimental approach to test the functionality of the -581 +50 bp fragment of the transferrin promoter, followed by the ability of the two loxP sequences to recombine in presence of the Cre protein allowing the expression of the HCV Core protein.

3.3.1 Functionality of the transferrin promoter in a murine cell line system

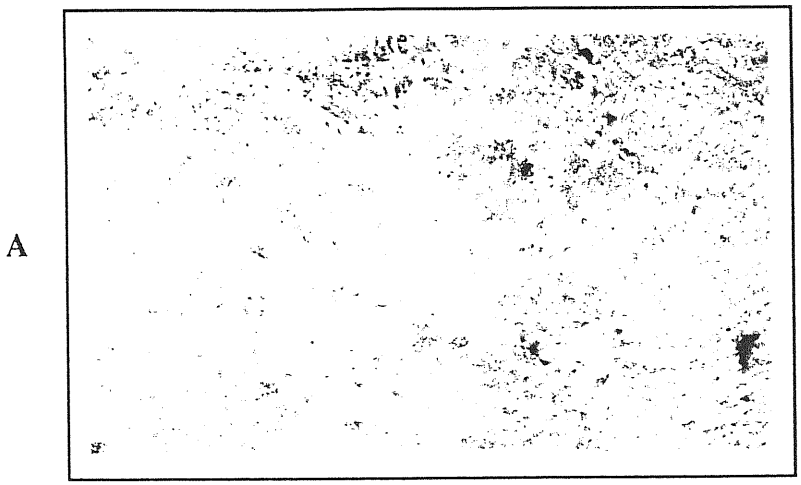
The functionality of the transferrin promoter was tested using transient transfection experiments in different cell lines. Since the -581 +50 bp fragment used in our plasmid displays a liver specificity we used cell lines of liver derivation in order to work in optimized conditions.

For this purpose we designed a new construct in which the -581 + 50 bp transferrin promoter was cloned directly upstream of the a viable ß-gal ORF coding region in a Bls vector. Transient transfection assays were then performed in two type of cells. Transfections were performed as described in material and methods.

The first type of cells transfected were the human hepatoma line HepG2. After 48 hours the cells were stained to evidentiate the ability of the transferrin promoter to activate the β-gal transcription. The results obtained were not optimal and very little staining could be detected. The low level of staining observed in this experiment prove that the mouse transferrin promoter was not so active in a human cell line.

Therefore, we then tested a mouse liver epithelial cell line (NMuli). The NMuli cells were transfected with the same construct as described above and after 48 hours they were stained to test the viability of the β-gal protein (Fig.3.3.1 panel A). As a positive control in all these experiments we used a Bls plasmid with the β-gal gene under the CMV promoter (shown in Fig 3.3.1 panel B), The ratio between total number of cells and positive Blue cells observed in each well showed that the efficiency of CMV promoter was at least 50 times higher than the efficiency of the transferrin promoter, and this owing to the fact that the CMV promoter is much stronger with respect to the transferrin promoter.

The third panel (Fig 3.3.1, panel C) represents a negative control in which no DNA was transfected but normal cellular staining was performed. The total absence of a blue cells in the negative control confirms the staining specificity of the viable β-gal enzyme.



A

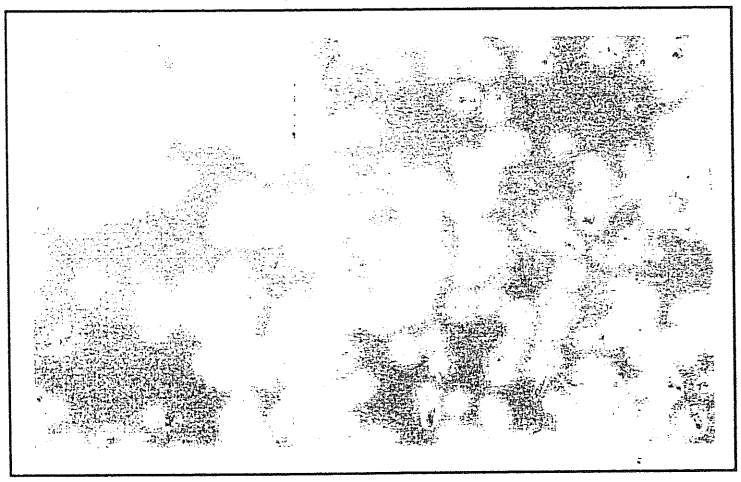
fragment
transf.prom.
(-581,+50bp)

Lac-Z gene

B

CMV prom

Lac-Z gene



C

Control cells.

Fig 3.3.1: Nmuli cells were transfected with a construct in which the Lac-Z gene was cloned downstream the transferrin promoter (A). The functionality of the promoter was tested using a functionality transfection assay. Panel (B) reports the transfection of a construct where the Lac-Z gene was cloned downstream the CMV promoter. In panel (C) the cells were not transfected.

Taken together, these experiments show that the fragment of the transferrin promoter is capable of driving mRNA production in transfection experiments. In particular, it is interesting to note that the same promoter fragment was more efficient in a mouse cell line than a human cell line confirming the earlier observations concerning the difference between the human and mouse transferrin promoters.

3.3.2 The expression of the β-gal protein in a cellular system

When the entire construct Bls 906 Core lox/Lac-Z was transfected in the NMuli cells we find a low number of positive blue cells indicating expression of β-gal. In the final construct a functional clone of Lac-Z was cloned without the use of further rounds of amplification that could have introduced mutations able to decrease the β-gal expression. In addition, the in frame design of the vector exclude any lack of frame that could be responsible of this low expression of the protein.

In order to clarify this observation we used a different plasmid in which the introduction of a T7 promoter allows the use of a Vaccinia virus system, thus increasing the efficiency of protein transcription in transient transfections experiment.

As shown in Fig 3.3.2 it is possible to observe that the presence of the 5′ UTR plus the first three aminoacids of the Core protein and the 34 bp of the lox sequence between the viral 5′ UTR and the Lac-Z gene decrease the expression of the protein in comparison with the efficiency of translation observed when the same Lac-Z clone was cloned directly under the T7 promoter.

A possible explanation of these results can be found in the work of Brown et al (Brown et al., 1992) in which they predicted that the secondary structure in the 3′ end of the 5′ UTR plays an important role in translational regulation by generating ribosome landing pads, or an IRES. The presence of extraneous nucleotide sequences in that region may dramatically alter the ability of these sequences to support internal initiation translation. More recently,Rijnbrant (Rijnbrand et al., 1996) shown

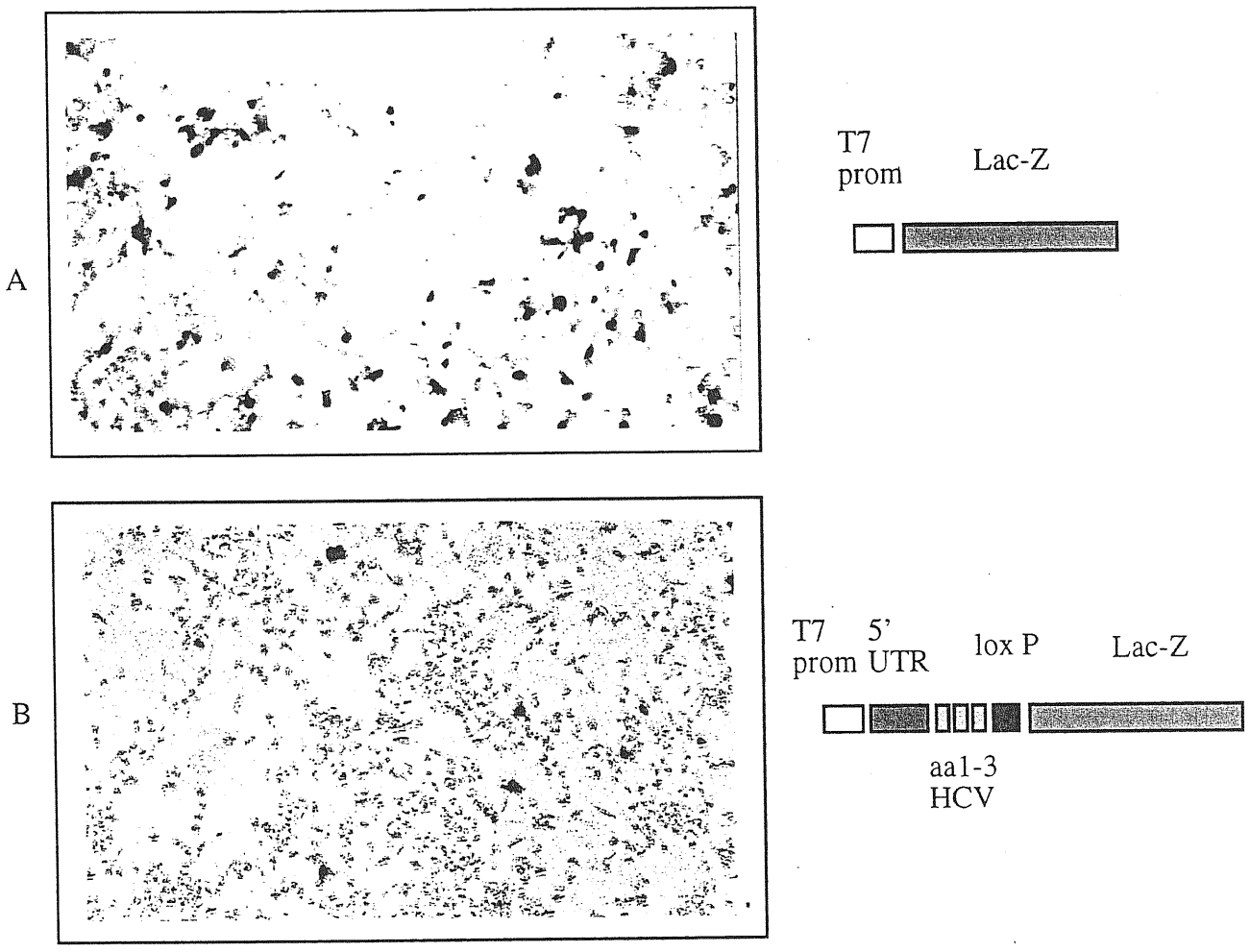


Fig 3.3.2: Two types of construct were transfected in Hela cells after previous infection with the vaccinia virus.
The Lac-Z gene of the first construct is localised directly downstream of the T7 promoter. (panel A). A strong blue signal was observed after staining the cells.
Panel B shows the transfection of a construct in which the 5' UTR , the first three aminoacids of the HCV Core protein and e 34 bp loxP sequence were inserted between the T7 promoter and the Lac-Z gene. A substantial decrease of β-gal expression was observed using this construct.

that the ribosomes are not able to initiate translation at a AUG codon located as close as 10 nucleotides downstream of the original position of AUG 342 in the 5′ UTR. Therefore in our system the presence of 45 nucleotides (lox P sequence plus the first three aminoacids of the Core protein) between the 5′ HCV UTR and the AUG of the Lac-Z gene could be responsible of the low protein expression observed.

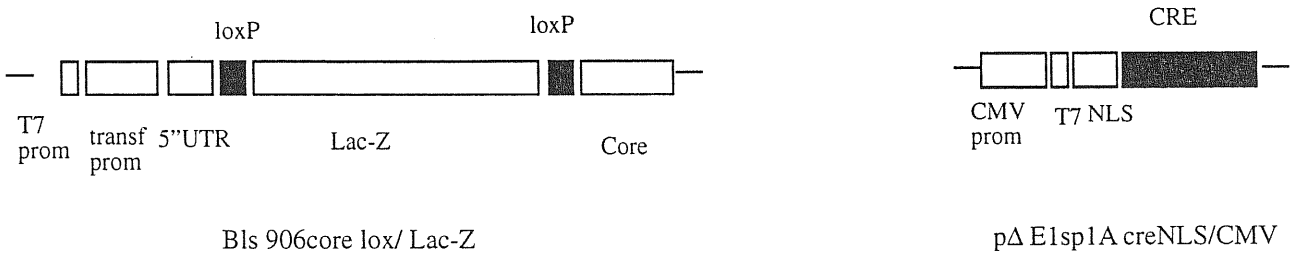
3.3.3 A cellular system was used to test the ability of a transient expression Cre protein to induce the recombination and the expression of the Core protein

A 3,439 bp-HindIII fragment of pBS185 containing the CMV promoter, the Cre ORF, and the methallothionein-I polyadenylation signal (kindly provided by Andres Muro, ICGEB, Trieste) was cloned BamHI/Hind III site into the polylinker of the plasmid pΔE1sp1A. The T7 promoter sequence was then added in order to allow the use of the Vaccinia virus system. In addition, a nuclear signal was placed upstream of the Cre ORF sequences in order to enhance the efficiency of recombination by increasing the concentration of the protein in the nucleus.

The presence of the recombinant Core protein expressed from Bls906Corelox/Lac-Z after the intended Cre dependent recombination was then tested by cotransfection experiments in different cell lines.

In the first approach the two plasmids Bls906Corelox/Lac-Z and pΔE1sp1A Cre NLS/CMV were cotransfected in Hela cells previously infected with Vaccinia virus, as shown fig 3.3.3.1 The presence of the Core protein was then demonstrated in western blot using a conformational monoclonal human antibody against its N-terminal region (aa 34-45). This antibody is called B12.F8 and was a kind gift from prof. Mondelli. The signal was developed using the ECL system(in order to increase the sensibility of the western).

Four different Cre clones (shown in lanes 2,3,4,5) were tested. The presence of the Core protein was observed in all cases at the expected MW of 22Kd. No Core protein was observed in cells transfected only with Bls906Corelox/Lac-Z in the



Bls 906core lox/ Lac-Z

pΔ E1sp1A creNLS/CMV

Cotransfection in Hela cells

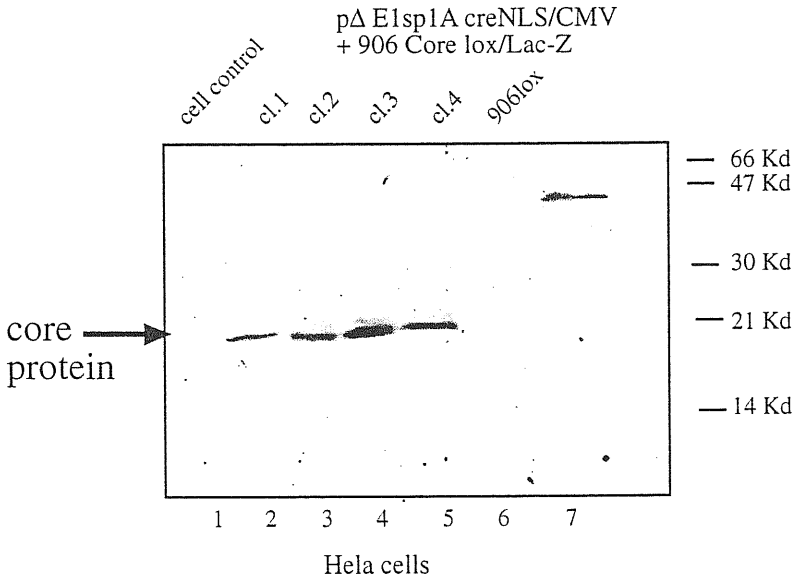


Fig 3.3.3.1. Western blot performed to verify the presence of the Core protein expressed after recombination in Hela cells culture. The upper part of the figure shows the two plasmids cotransfected in order to allow the recombination and consequenly Core protein expression . The cells were infected before cotransfection using a vaccinia virus system. The Core protein was identified using a monoclonal antibody B12.F8 against the N-terminal region of this protein.

absence of the Cre protein (lane 6) and no Core protein was detected in untransfected cells (lane 1). Lane 7 shows a positive control.

A good level of recombination was observed in this experiment, confirming the association between the lox P sequences and the Cre protein. Moreover, the expression of the Core protein confirms the functionality of the recombination of our vector in a cellular system.

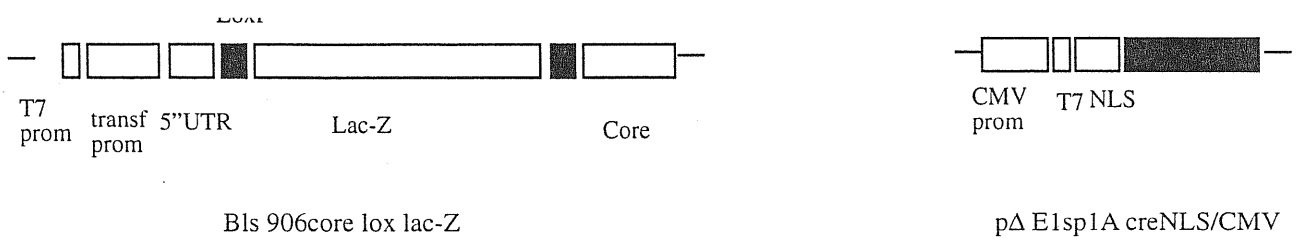
Since these results were obtained using the T7 promoter present in both plasmids we performed a second set of cotransfections in NMuli cells to confirm the presence of the Core protein in a system under the control of the transferrin promoter.Lane 2 of fig 3.3.3.2 shows that also in this case a recombinant HCV Core protein can be observed. As expected, the expression of the protein is lower in comparison with the amount obtained when the Vaccinia virus is present (lane 4). Again, the Core protein was not expressed in absence of the Cre protein (lane 3).

In conclusion, the results of these experiments were very promising in the light of a future in vivo application, also because for the study of a pathogenic mechanism a very strong expression may be toxic whilst a low constitutive expression ideal.

3.3.4 A cellular system was used to test the presence of the Core transcripts after a transient Cre mediated recombination

To test the presence of Core protein RNA after the Cre dependent recombination in the NMuli cells we also performed a Northern blot using total RNA extracted from this transfection experiment.

Fig 3.3.4 panel A shows a northern blot obtained from total RNA extracted after 48 hours after the transfection. A positive signal lower of the ribosomal 18S RNA was observed in lane 1, where we loaded the total RNA extracted from the cells cotransfected with the two constructs Bls906Corelox/Lac-Z and pΔE1sp1A Cre NLS/CMV. This band is of the expected size since the excision of the Lac-Z gene produces a RNA of 1500 bp long Total RNA extracted from cells only transfected



Bls 906core lox lac-Z

pΔ E1sp1A creNLS/CMV

Coransfection in Nmuli cells

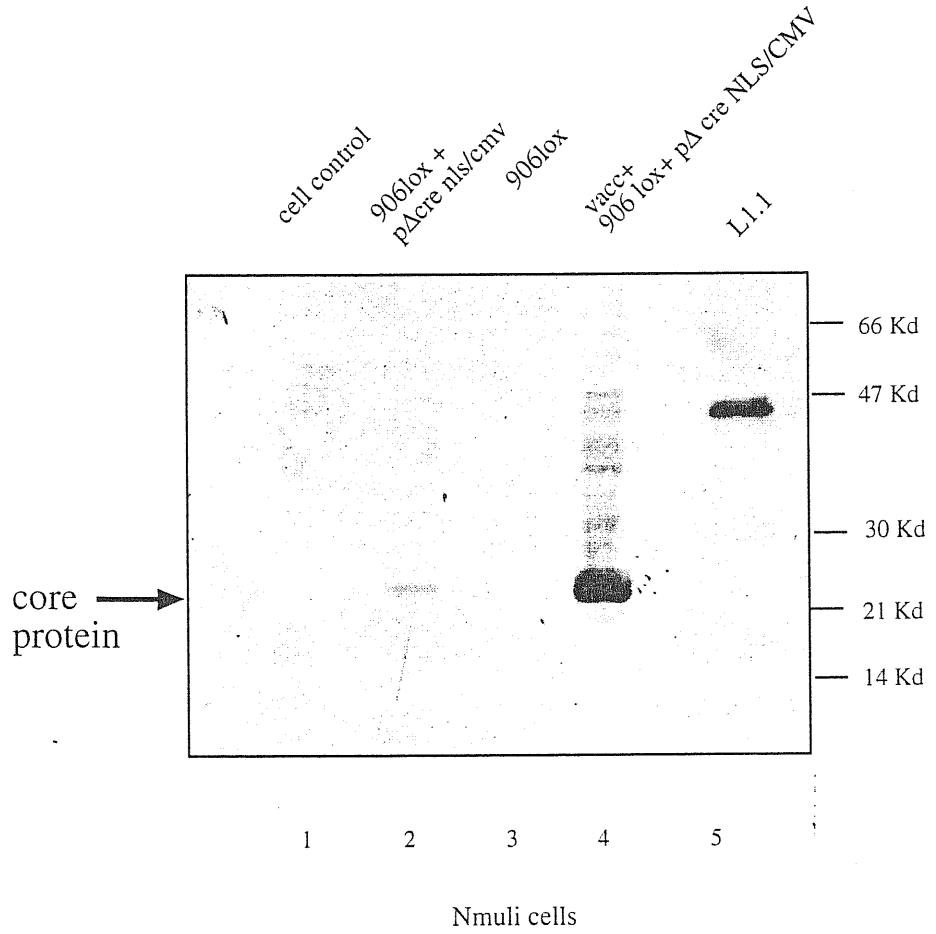


Fig 3.3.3.2: Western blot performed to verify the presence of the HCVCore protein after recombination in NMuli cells.The presence of the protein was tested using a monoclonal Antibody against the N-terminal region of the Core. The western blot was developed using an enhancer chemiolunimescence technique. (ECL).

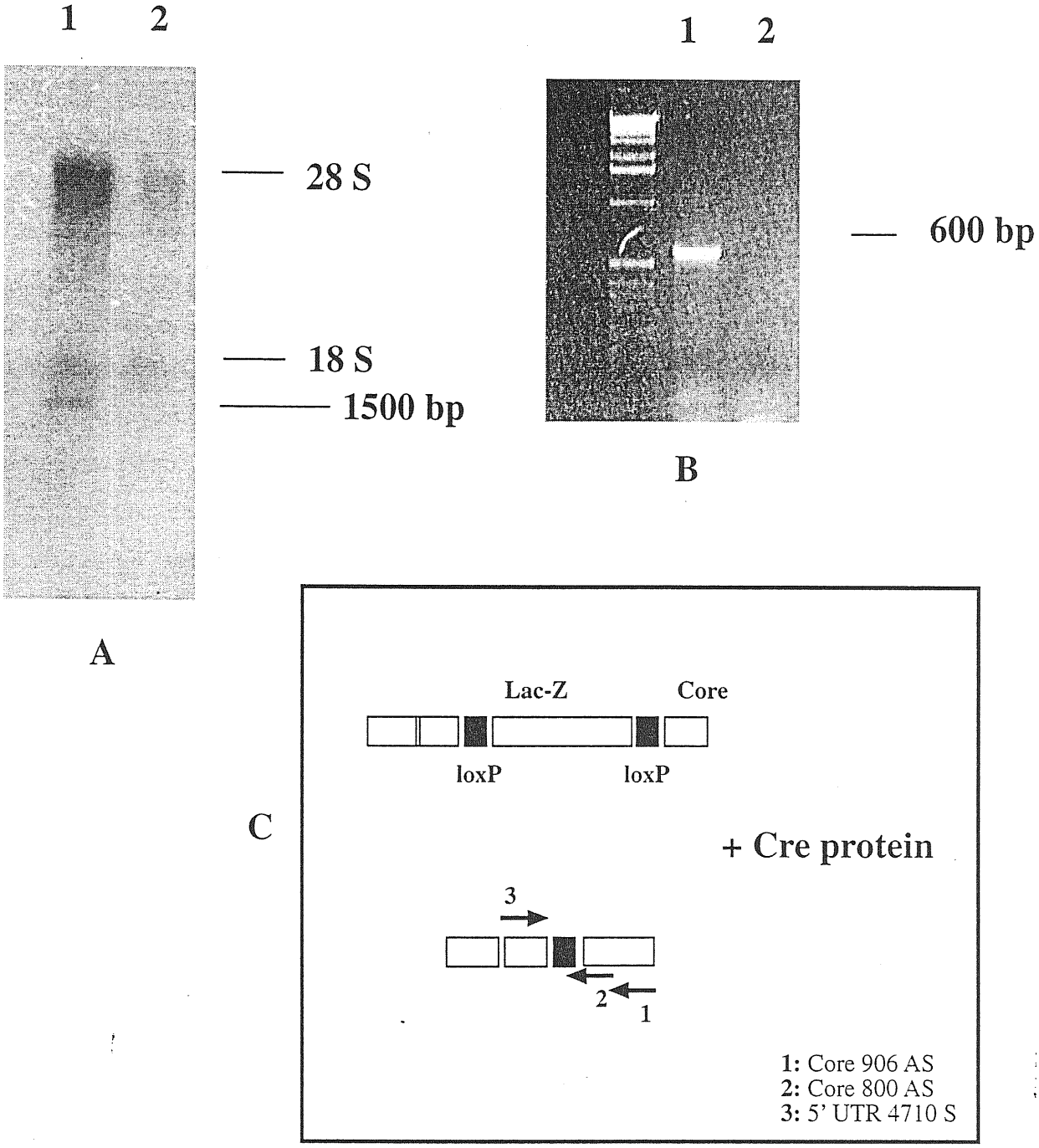


Fig 3.3.4: Panel A was shows the northern blot performed on total RNA extracted from NMuli cells cotransfected with the construct Bls906 Core lox/Lac-Z and the construct PΔE1sp1A (lane 1). The total RNA extracted from cells transfected only with the construct Bls906 Core lox/Lac-Z was used as negative control (lane 2). A BamHI/XbaI fragment of the HCV Core protein was used as radioactive probe.
In the panel B the result of a RT-PCR performed on the some cellular RNA is shown. The band obtained on the total RNA of NMuli cells contransfected with the two constructs presents the expected molecular weight (lane1). No positive results were obtained using RT-PCR on the RNA from cells transfected only with the Bls906 Core lox/Lac-Zconstruct (lane2). In the panel C was shown the position and the nome of the primers used for the RT-PCR.

with the construct Bls906CoreLox/Lac-Z alone does not give any positive signal (lane 2). The membrane was then hybridized with a labelled ^{32}P BamHI-Xba I Core fragment as probe.

In order to confirm this result we performed an RT-PCR on total RNA after previous treatment with DNase. The primers used for the reverse reaction (Core 906 AS) were localised on the 3′ end of the Core protein. The two primers utilised for the PCR amplification (Core 800 AS and 5′UTR 4710 S) are localised out of the two loxP sequence(Fig 3.3.4 panel C). A band of the expected size (Fig.3.3.4 .lane 1 panel B) was obtained from the RNA extracted by the cells in which the recombination had occurred. No positive signal was obtained from cells in the absence of Cre protein (Panel B lane 2).

SUMMARY OF THE PRELIMINARY EXPERIMENTS

a) the -581 +50 bp of the transferrin promoter is able to drive the expression of the Core protein in a mouse cell system. This confirms the suitability of the promoter fragment chosen for the vector that will be used in the generation of the transgenic animals.

b) the expression of the Core protein is dependent on the presence of the Cre protein. Only when the recombinase is present the Core protein can be synthesized. Moreover, the introduction of the 34 bp of the loxP sequences in the ORF of the nucleocapsid protein of the hepatitis C virus doesn't alter its conformation as demonstrated by the fact that it is still recognised by a conformation specific Core antibody (B12.F8).

3.4 THE TRANSGENIC ANIMALS

3.4.1 Generation procedure

The micro injection of the DNA directly into the pronuclei of fertilised mouse eggs causes a stable chromosomal integration of the transferred gene in 10-40% of resulting mice. Normally, 200-500 copies of the transgene are introduced into the pronucleus of fertilised mouse eggs and they are usually found in a single chromosome in a head to tail organisation. If the integration takes place at the one-cell stage it gives rise to animals which are transgenic in all cell tissues. Integration of the transgene at the two-cell stage probably results in founder animals which still have the transgene in every tissue but only in a variable proportion of cells. Finally, integration in later stages results in the presence of the gene in some tissues and not in others.

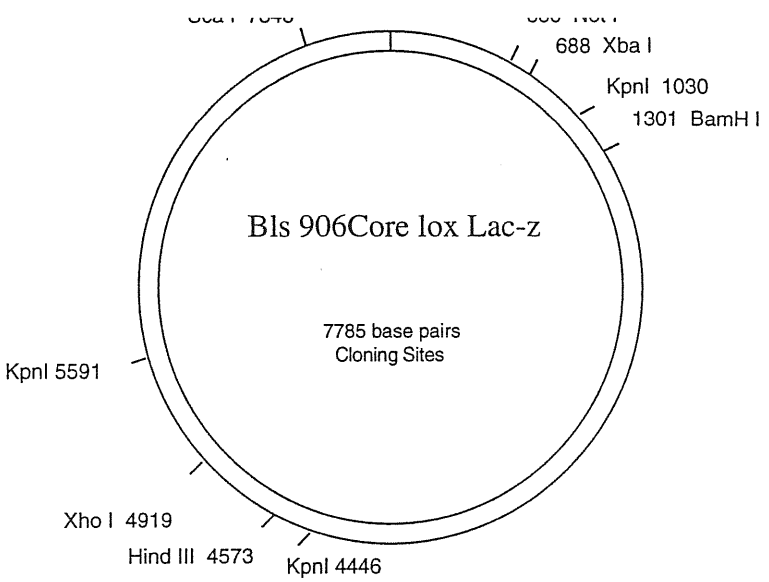
The purified vector Bls906Corelox/Lac-Z was then injected into fertilised mouse oocytes using standard techniques and the transgenic mice were produced This step was not carried out by the candidate.

However, before this event took place a quantitative southern blot experiment had to be set up in order to identify the transgene in the animals produced. The general procedure used for our southern blot is described in the Material and Methods section.

The DNA fragment (4239bp) to be mixed with the genomic DNA was then prepared by digesting the Bls906Core lox/Lac-z construct with the restriction enzyme XhoI and NotI. The enzyme NotI was used in place of XbaI since in our construct this restriction enzyme site is inhibited in bacteria by methilation (the most important restriction sites used in the southern blot are represented in the map of the fig 3.4. 1. panel A).

DNA of a wt mouse (5ug/lane) was digested o/n with the same two restriction enzymes and the two DNAs (plasmid and mouse) were then mixed together in order to simulate the presence of 1, 5 and 10 copies of the transgene. A

A



B

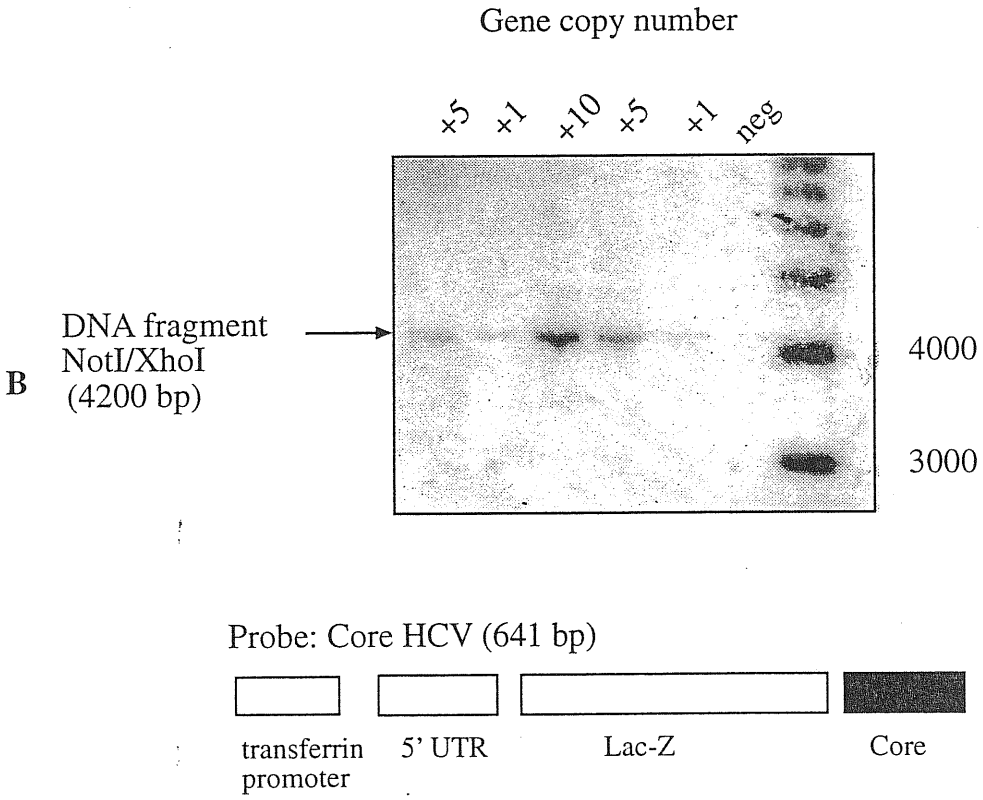


Fig 3.4.1: Panel A was shows the graphic rapresentation of the construct used for the production of transgenic mice. The most important restriction enzymes size are indicated.
Panel B shows the southern blot used in order to identify the minimal gene copy cell equivalent present in the transgenic animals.The BamHI/XbaI Core DNA fragment used as radioactive probe is indicated as a black box.

fragment of 641 bp corresponding to the BamHI/XbaI Core region excised from the Bls906Core lox/Lac-z construct was used as a radioactive probe as schematised in Fig 3.4.1. panel B.

The southern shown in Fig 3.4.1 contains in lane 1 the signal of 5 gene/copy cell equivalents of the 4239 bp fragment, and in lane 2 the signal of 1 gene/copy cell equivalents in the absence of wt mouse DNA. Lanes 3, 4 and 5 show the signal obtained after mixing the DNA of wt mouse with respectively 10, 5 and 1 gene/copy equivalent of the XhoI/NotI of the plasmid fragment. Lane 6 represents the negative control where only the wt mouse DNA was loaded. As we can see in this figure the presence of wt genomic DNA doesn't inhibit at all the signal obtained from the XhoI/NotI fragment excised from the Bls906Corelox/Lac-Z vector.

At the same time we performed an alternative route to mjcroinjection for generating a second type transgenic mice using Totipotent Embryonic Stem cells.

The cells can be transfected with a gene (by electroporation) and a selectable marker such as neomycin resistance. Stably transfected cells line are then used to generate chimeric embryos. Therefore, we electroporated a variant of the construct Bls906Core lox/Lac-Z in which the 5′ UTR of the genotype 1 was replace with the 5′ UTR of the HCV genotype 3. Previous in vitro study has confirmed the difference in the translation initiation ability of 5′ UTR belonging to genotype 1 and 3 (Buratti et al., 1997) and our interest was focused on the possibility to have two different transgenic animal models able to increase these preliminary results in an in vivo system.

3.4. 2 The transgenic lines

Twelve founder lines containing the Bls906Corelox/Lac-Z construct were sent to our lab (a list of each founder is reported in Table 3.4)

F1 mice were then obtained from each founder using successive back crossing with wt CD1 mice and these mice were then mated with each other in order to amplified each line. It was interesting to note that the progenies of the line 8581

carrying the transgene construct were always male animals. As the founder of this line was a male this fact prompted the hypothesis that in these animals the transgene was localised on the Y chromosome.

The screening for positive transgenic animals was routinely analysed by PCR using primers located respectively in the Lac-Z gene (Lac-Z 1509 S) and in the HCV Core sequences (Core 906 AS). These primers amplify a fragment of 600bp as shown in Fig3.4.2 Panel A. A Mendelian proportion of positive and negative mice was found after each mating.

3.4.3 Transgene structure

A factor which principally contributes to regulate the transcription of a transgene is the number of copies in which this gene is integrated in the genome. Since a PCR amplification is unable to discriminate accurately the copy number of a gene present in each line we therefore used a comparative southern blot approach to estimate the number of gene copies in each founder line.

In this kind of experiment around 15 µg of genomic DNA was extracted from the tails of transgenic animals and digest with the enzyme KpnI that cuts in the transgene. A radiolabeled probe was then used to detect the presence of the transgene. This probe is a KpnI-KpnI fragment of the Bls906Corelox/Lac-Z construct containing the transferrin promoter and the 5′ UTR region (as can be seen in fig3.4.2 panel B). The strategy is to compare the intensity of this band with the signal of a gene present in the animal in only one copy number. In our case the use of this probe allows the comparison of the transgene signal with the corresponding signal belonging to the native transferrin gene (present in only one copy number in the mice) but keeping in mind that the signal corresponds to the endogenous gene present on two alleles. The labelling intensity of the two bands in a genomic southern (Fig 3.4.2 panel B) was then analysed using a phosphoimager which allowed us to calculate the copy number of the transgene in all transgenic lines (reported also in table 3.4). This number can be affected by some errors due to the

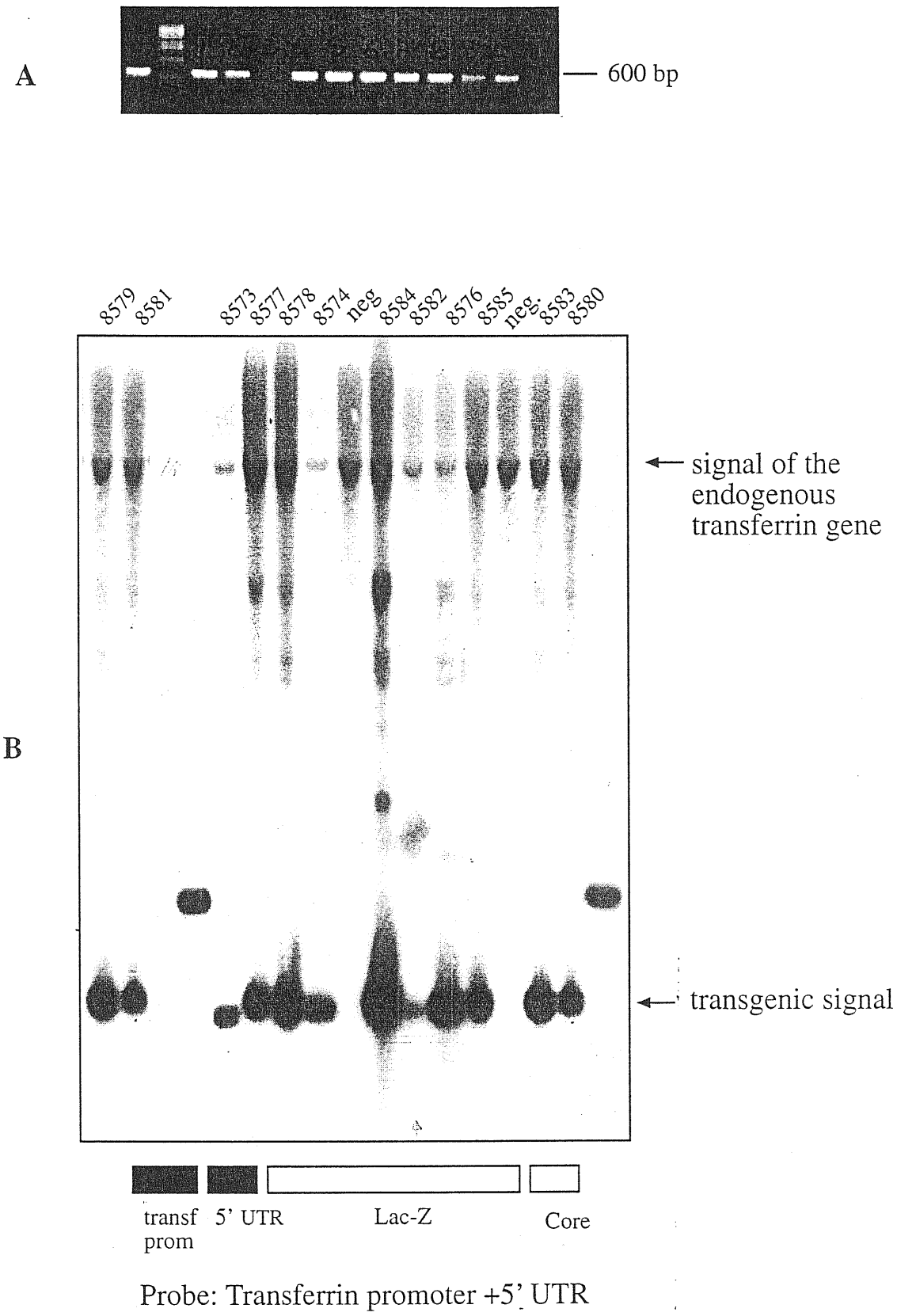


Fig 3.4.2: Panel **A** is shows the result of the PCR peformd on the genomic DNA to discriminate the positive transgenic mice.For this amplification we used two internal primers annealing on the HCV 5'UTR and the Lac-Z gene of the construct (Core 906 antisense and lac-Z 1502 sense)
Panel **B** rapresents a genomic southern blot used to identify the transgene copy number present in the mice genome of each lines.
The probe used in this experiment is highlighted as a black box.

high intensity of the transgenic bands in some cases but, nonetheless, gives a reasonable estimate of the approximate number of transgene copies present in each mice genome.

Founder	copy number
8573	20
8574	30
8576	35
8577	7
8578	10
8579	10
8584	30
8581	8
8582	3
8583	10
8580	8
8585	10

Table 3.4.: In the column on the left we report the designation of each different founder lines of transgenic mice, whilst on the right the deduced copy number of transgene present in each respective genome.

3.4.4 Transgenic expression

A second factor that profoundly influences the level of expression of a transgene is its site of integration in the genome. In our experiments it was already evident that one of the transgenic line carried out the foreign gene on the Y

chromosome. Since this chromosome is almost inactive the 8581 line was not be expected to display any transgene expression.

Originally, our plasmid had been intentionally designed so that before the introduction of the Cre protein the expression of the β-gal protein should have been observed in those animals which were actively transcribing the construct. Therefore, this protein would have represented the marker for the identification of those lines which displayed the highest levels of transgene expression. The liver of each transgenic lines was stained following the procedure reported in material and method. As a positive control of the experiment we used the transgenic mice C57BL/6J-TgR(Rosa26)26Sor (Rosa). These animals costitutively express the Lac-Z in all tissues (Friedrich and Soriano, 1991) and in fact, as shown in Fig. 3.4.4, the blue colour of the "Rosa"mice is very clear. On the other hand, no blue staining could be observed in the liver of our mice. Probably the low β–gal expression previously observed in an in vitro system (see section 3.3.2) was not sufficient to be detected in the animal model. In addition the expression of the β–gal protein was checked also in other tissues as lung, hart, kidney, spleen and brain. Also in these case a β–gal protein expression was not observed

The impossibility to utilised the β-gal enzyme viability as a marker for the translation has forced us to analysed the level of transcription present in all the twelve transgenic lines using different techniques.

3.4.4.1 Northern blot

We then analyzed liver specific transgenic expression of Bls906Corelox/Lac-Z mRNA using Northern blots (for the standard procedure see material and method). These studies were initially negative after using different DNA probes excised from the Lac-Z gene and the Core sequence. In particular, 20 ug of total liver RNA was extracted from the different founders mice and analysed using eight different β-gal probes and two Core region probes. As positive control for the β-gal probes we used the total RNA extracted from the liver of the transgenic C57BL/6J-TgR(Rosa26)26Sor

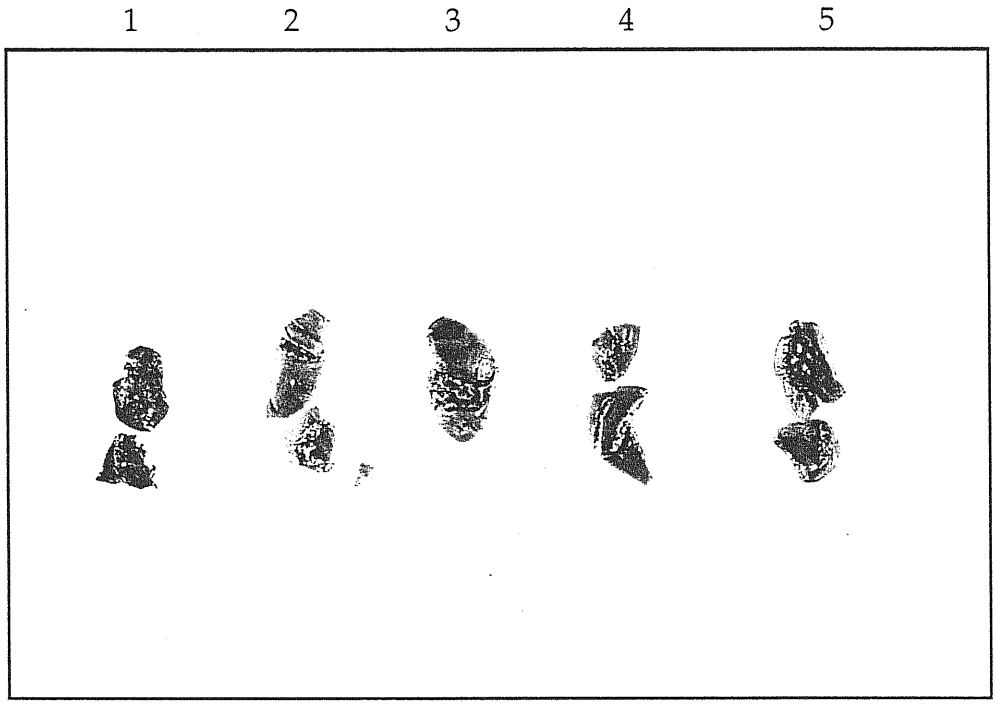


Fig 3.4.4.: The liver of the transgenic mice belonging to the three lines 8574, 8576 and 8584 were stained to test the presence of a b-gal protein in vivo (lane 2,3,4). As positive controll we used the transgenic mice C57BL/6J-TgR(Rosa26)26 Sor,(lane 1) which constitutively express the Lac-Z gene and as negative controll the liver of a Swiss mouse (lane 5).

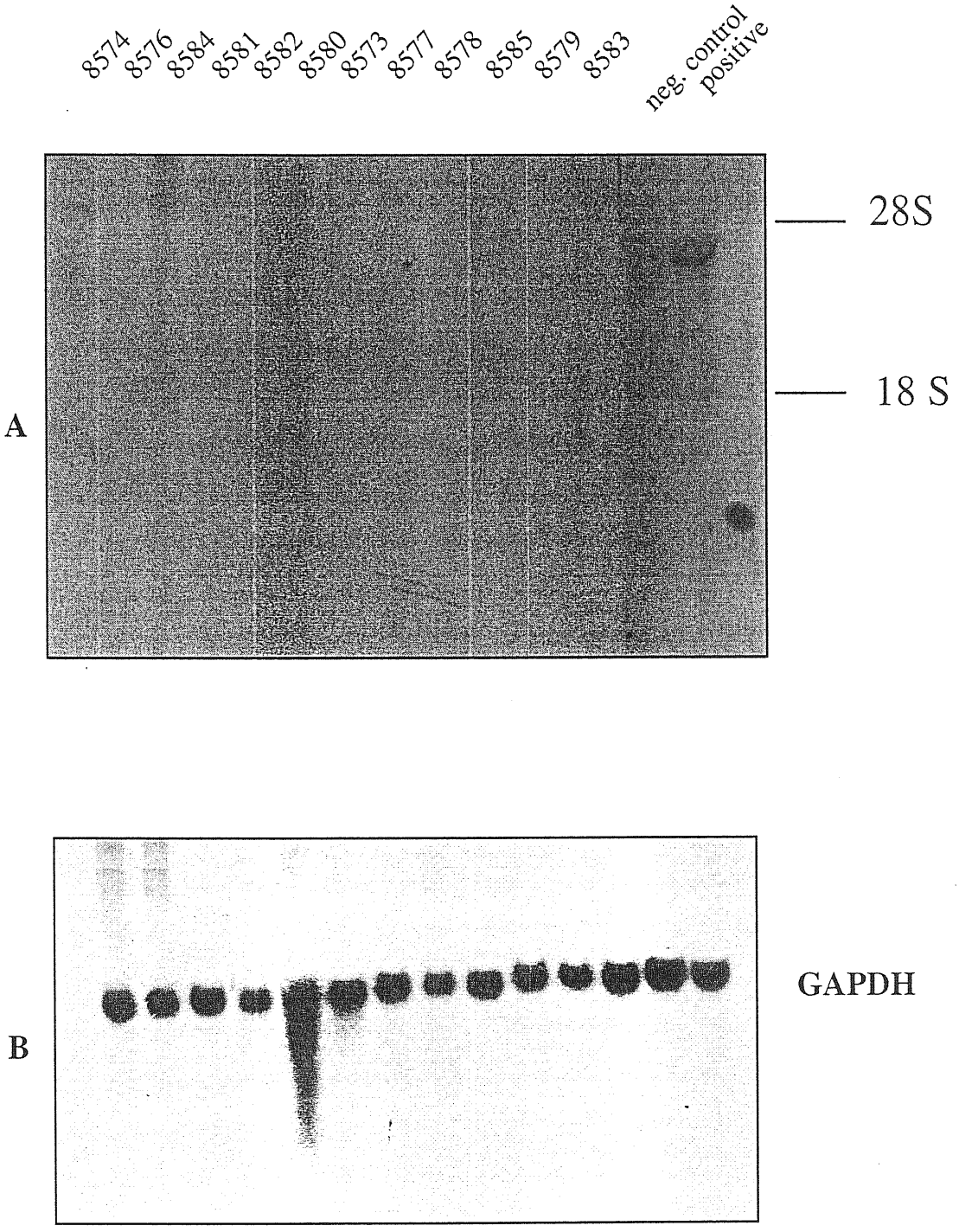


Fig 3.4.4.1: Northern blot analysis on transgene expression in liver. Total RNA from the liver of all the transgenic lines and from two control animals was separated on a 1% agarose gel containin formaldehyde and transferred to nylon membrane. It was probed by a ^{32} P labeled Lac-Z gene amplified fragment (panel A).
The same membrane was also probed by a ^{32}P labeled GAPDH probe to control for gel loading (panel B).

mice. As negative control we used the total RNA extracted from the liver of wt mice. However, in all experiments too much aspecific signals were obtained and therefore we changed strategy to obtain a specific probe for the β–gal expression.

Using the two primers, GC 105 and GC 106, localised on the Lac-Z gene sequence we amplified a fragment of 500bp that was then tested as probe in the Lac-Z positive mice. This time, a specific signal was finally obtained for our positive controls and the same probe was then used to test the presence specific mRNA in the transgenic lines, as shown in Fig 3.4.4.1 panel A. The exact size of this RNA could not be predicted because these transgenic mice were obtained using a gene trap strategy (Friedrich and Soriano, 1991). Accordingly, the wild type mice used as a negative control didn't present any kind of aspecific signal. Each membrane was then reprobed by a ^{32}P labelled GAPDH probe to normalised the amount of RNA loaded (Fig 3.4.4.1 panel B). No transcription signal was found on the total RNA extracted from the liver of all the 12 transgenic lines and this data suggest the complete absence of transgene transcription in vivo. The same negative results were observed when the northern blot was performed on all the other tissues analysed.

3.4.4.2 RT-PCR and RACE

A second experimental approach was then used to confirm the results obtained with the northern blot based on the RT-PCR technique. The total RNA extracted from mice liver was reversely transcribed as described in Material and Method and a synthetic cDNA was obtained using different antisense primers. As primers we used both specific internal primers (Core 718 antisense) localised at the 3' end of the Core sequence but also oligo dT primer and a set of random primer. The two primers used for the PCR amplification are localised respectively on the Core sequence (Core 800 AS) and on the β–gal sequence (Lac-Z 1502 S).

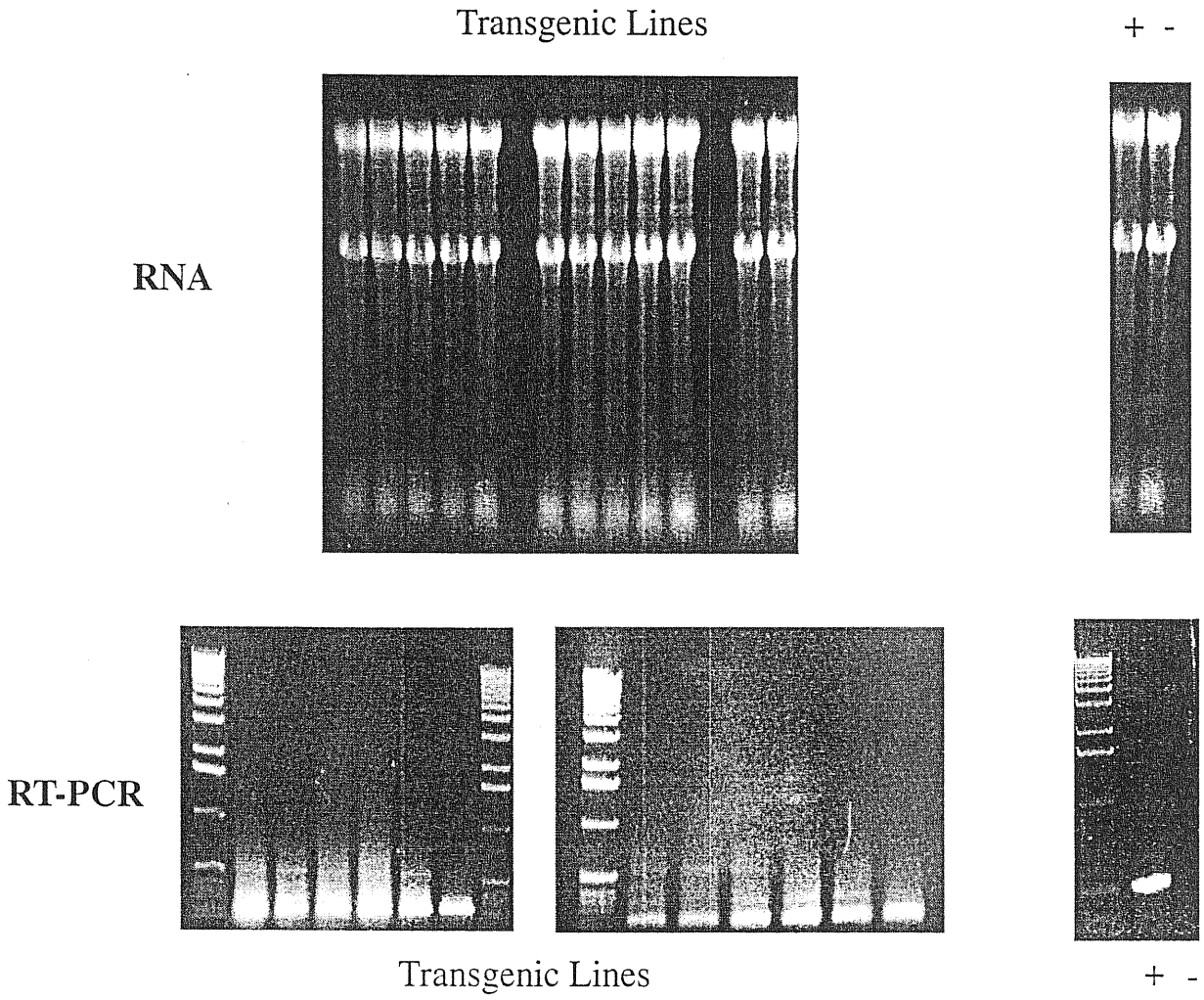
Since the extraction of total RNA can be contaminated from genomic DNA the total RNA was in all cases previously treated with the DNAse enzyme in order to digest any genomic DNA. Furthermore, each step of cDNA synthesis was performed

with and without the presence of the Reverse Transcriptase enzyme. Finally, the good quality of the RNAs extracted from transgenic mice was tested using a set of primers able to amplify an endogenous gene such as GAPDH or β-actin. Also for these experiments we used the cDNA obtained from the RNA of the C57BL/6J-TgR(Rosa26)26 Sor mice as a positive control of RT-PCR amplification. In this case we used an oligo dT primer for the cDNA synthesis and a set of primers on the β-gal (GC 105 sense and GC 106 antisense) for the PCR amplification. (Fig 3.4.4.2).

However, no positive data were obtained from this first the RT-PCR analysis performed on the total RNA of the transgenic mice (Fig. 3.4.4.2) also using a second round of PCR (nested PCR) (using the primers Core 906 AS and Lac-Z 1380 S) in order to increase the sensibility of this approach.

One possible explanation for this is that the DNAse treatment, an essential step in RT-PCR, can damage the RNA. Therefore, reverse transcription of not very abundant mRNAs can be inhibited by this enzyme.

Therefore, in order to avoid the DNAse step a 3' Race approach was used (see material and Methods for details). With this technique it is not necessary eliminate genomic DNA because the cDNA is obtained using a particular type of primers which contain a Oligo dT sequence followed by a generic sequence named "anchor" (RACE 3'dTIB AS). The oligo dT is then able to prime only on a RNA polyadenilation site during the cDNA synthesis and the PCR amplification will be performed on this cDNA using an antisense primers whose nucleotide sequence is complementary to that of the anchor present on the primer used for the Reverse Transcription reaction (RACE anchor AS). As sense primer we then used specific internal primers priming on the Core sequence. RACE 799 S and RACE 896 S).As usual, the positive control of this technique represented by the total RNA extracted from the liver and the brain of the C57BL/6J-TgR(Rosa26)26 Sor mice. Therefore, cDNA was synthesized using the oligo dT-anchor primer and first of all the GAPDH endogenous gene was amplified to test both the functionality of this approach and the quality of the RNA. Both amplifications of the GAPDH gene from brain and liver and the amplification of the



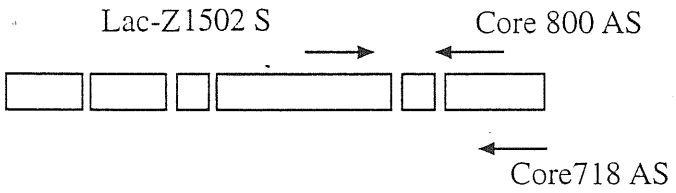


Fig 3.4.4.2: The RT -PCR performed on the total RNA extracted from the liver of the transgenic mice belonged to all the lines does not show anypositive signal. (RT-PCR panel) The primers used are reported in the figure.
For the RT reaction we used the internal primer Core 718 AS and for the PCR reaction we used two primers localised on the Core protein (Core 800 As) and on the Lac-Z gene (Lac-Z 1502 S). A cDNA obtained from the total RNA of the C57 BL/6J-TgR9 (Rosa 26)26Sor mice was used as a positive control of RT-PCR amplification. In this case we used an oligo dT primer for the cDNA synthesis and a set of primers on the β-gal for the PCR amplification.
As negative control we used the total RNA extracted from normal Swiss mice.

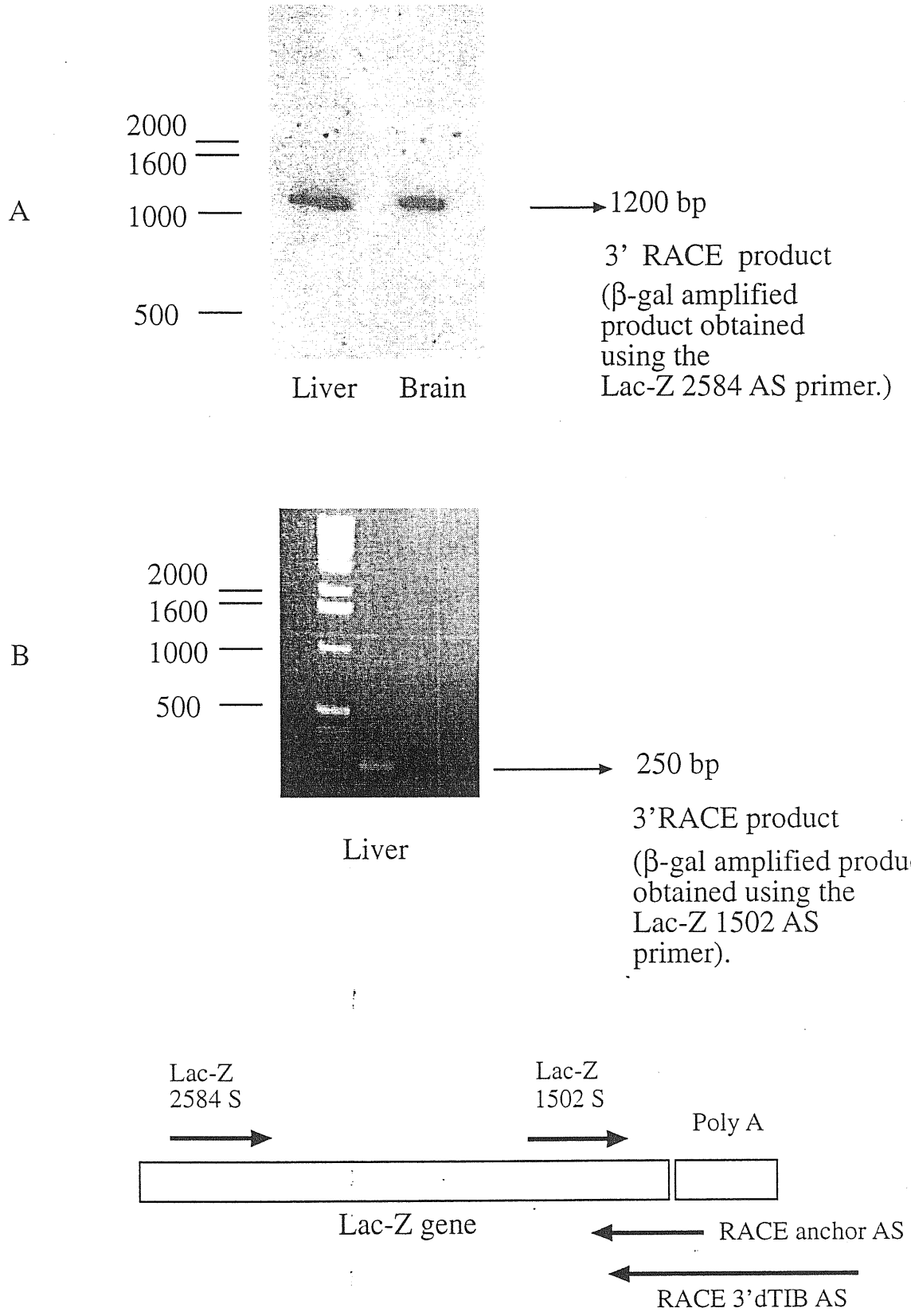


Fig 3.4.4.3 : The total RNA extracted from the liver and the brain of the C57 BL/6J-TgR9 Rosa 26)26Sor transgenic mice was reversely trascribed using a oligo dT RACE approach. The PCR step was performed using different primers. As antisense primer we used the same Race anchor primer.
As sense primer we used primers localised either near the 5′ region of the Lac-Z gene(Lac-Z 2584 S)(Panel A) or near the 3′ end of the same gene (Lac-Z 1502 S) (Panel B)

β-gal fragment using different internal primers (Fig 3.4.4.3) gave positive results. The name and the sequence of all primers used for each amplifications are reported in the table 2.1 in the Material sand Method section. However, also in this case it was impossible to have any amplificated product from liver RNA of the transgenic mice.

Since at the same time we had already developed the Adenovirus vector to shuttle the Cre protein in the transgenic mice we decided to perform the in vivo recombination on the transgenic mice since it was indeed possible that the chromosomal rearrangement induced by the Cre recombinase action would activate transcription of the resulting HCV Core mRNA from the transferrin promoter at least in the minimal amount required for our purpose.

3.5 THE ADENO/CRE SYSTEM

Although a defective recombinant Adeno/ Cre virus was already published by Anton(Anton and Graham, 1995), it was not available and therefore it forced us to create a new one.

3.5.1 The Adenovirus: characteristics

The strategy of gene-inactivation is based on causing the disruption of the promoter/coding region structure required for expression which can be achieved through a site-specific recombination process of excision. This approach was successfully used for the first time in the conditional inactivation of a gene in a particular tissue of transgenic mice throughout following the specific expression of Cre protein (Lakso et al., 1992).

Considering that our objective also included the delivery of Cre recombinase in our transgenic mouse tissues, we decided to use a system that had already been successful to reach this aim. Therefore, we decided to use a human adenovirus (Ad) vectors which has been used extensively for heterologous gene expression in mammalian cells (Berkner, 1992) and have therefore attracted considerable attention as potential recombinant vaccines (Graham and Prevec, 1991; Prevec et al., 1990) or for use in gene therapy (Le Gal La Salle et al., 1993; Ragot et al., 1993; Stratford-Perricaudet et al., 1992).

The advantages of using this system are numerous. Most importantly, the 36000-bp double stranded DNA genome of Ad is relatively easy to manipulate by recombinant DNA techniques (Graham and Prevec, 1991) and the genome does not undergo rearrangement at a high rate. In addition, the viral particles are relatively stable and the virus replicates efficiently in permissive cell, enabling the production of high-titter viral stock. In non dividing , non permissive cells the viral genome may persist as an episome and continue to express for long periods. Finally, it should be pointed out that in general the defective Adenovirus vectors do not integrate into the

host chromosomes and in these cases the expression of the foreign gene mediated will be transient.

The Adeno virus genome encodes at least 30 mRNA species and is organised into several early (E1-E4) and late transcriptional regions (L1-L5), each of which plays a specific role in the viral life cycle. Adenovirus types 2 and 5 have been the ones most widely studied in terms of their genomic organization, and as a consequence most of the Adenoviral vector constructed are based upon these two viral serotype. Although adenoviral vectors with different genomic organisation have been developed the majority falls into two categories based upon the insertion of the exogenous expression construct into either E1 or E3 regions (Berkner, 1988). The basic viral backbone in each class of vectors involves the deletion of the original adenoviral sequences and the replacement of these regions with an expression cassette containing the gene of interest. Since the E3 region of the virus is not essential for viral replication the vectors constructed by deletion and replacement of the E3 sequences are replication-competent (Kelly and Lewis, 1973). On the other hand, E1 is a gene essential for Ad replication and therefore the replacement of this region allows the creation of a replication-defective vector. Deletion of essential Trans-acting regions such as E1 restricts the growth of these viruses to cells expressing this gene. This can be achieved in two ways: by using a helper virus complementing the defective genome or by introducing the corresponding region of the Adenoviral genome in the chromosomes of the cells used to produce and amplify the virus (complementation cell lines). Presently, this is the preferred method and several cells lines have been developed which express the E1 protein, such as the 293 cell line which contains E1 region of Ad5 (Graham et al., 1977). However, it must be noted that two sub regions within the E1 region must be retained if the vector is to be able to replicate in 293 cells (Hearing et al., 1987). Nonetheless, deletion of up to 3.2 kb can be made in the E1 region without compromising the ability of the defective virus to replicate in 293 cells.

A disadvantage of using Adenoviruses compared to other viral system is their limited cloning capacity. Traditional adenovirus vectors contain a 3 kb deletion in the E1 region and a 1.8 kb deletion in the E3 sequence. In fact, adenovirus capsids can package genomes not exceeding 5% of the normal size of the wild-type genome, thus making these viruses able to package a foreign genetic material for a maximum of 8 kb (Graham and Prevec, 1991). Recently, research has been aimed at improving this cloning capacity. (Bett et al., 1994; Bett et al., 1993; Graham and Prevec, 1991).

Two ways have been described to obtain replication-defective Adenovirus vectors: direct ligation into Adenoviral DNA or homologous recombination in vivo. Recently, Graham and colleagues have reported a modification of this procedure whereby the transfer plasmid is cotransfected with a circular form of adenoviral DNA (such as pJM17) in which a bacterial origin of replication and ampicillin resistance gene have been inserted. The homologous recombination between the two plasmid results in the substitution of the bacterial sequences in pJM17 by the expression cassette into and adenoviral genome that can be packaged efficiently into virions (McGrory et al., 1988).

3 .5.2 Strategy used to obtain the recombinant Adenovirus Ad CMV NLS/Cre

Our decision to use an adenovirus vector was justified by the fact that previous reports had already shown that even transient expression of the Cre protein was sufficient to excise the lox-P flanked sequences in the chromosome (Sakai et al., 1995).

The first step was to clone into the Bam HI/Hind III site of the shuttle plasmid pΔE1sp1A a fragment of plasmid PBS 185 which contains (Sauer and Henderson, 1990) the CMV promoter, the Cre ORF, a nuclear signal, and the methallothionein-I polyadenilation signal (Bett et al., 1994) The inclusion of the nuclear signal was performed with the aim to enhance the presence of the recombinase in the nucleus, thus increasing the percentage of final recombination. The resulting plasmid containing the Cre expression cassette flanked by the Ad E1 sequences was then

cotransfected in 293 permissive cells with plasmid PJM 17 to obtain the recombinant Adenovirus. The PJM17 plasmid consists (McGrory et al., 1988) of a complete Ad5 genome with an E1 insert of pBR322 which exceeds the packaging constraints of Ad5. This fact implies that infectious progeny will result only by recombination with the shuttle plasmid containing the foreign gene. Fig 3.5.2.1 shows the homologous recombination that should take place between the two plasmids in 293 cells.

The identification of single clones of recombinant Adenovirus was performed using a dot blot hybridisation assay with a probe consisting of a fragment of the Cre recombinase cDNA (an example of this screening assay is shown in Fig 3.5.2.1). It should be noted that the first dilution shows a weaker positive signal when compared to the one observed in the successive two higher dilutions. This observation is in agreement with the fact that in the first dilution (10^{-1}) a great number of infected cells had died and was lost after washing the wells. We then performed a limited dilution assay to select those clones to be amplified in 293 cells and after large scale preparation of the virus the titter of the stock eventually calculated to be $1x10^{8}$ (particles/ml). The recombinant Ad Cre NLS/CMV was amplified , centrifuged and filtered in order to eliminate possible bacterial contamination. The steps involved in the production of a defective recombinant Adeno/Cre virus are described in materials and methods.

3.5.3 Functionality of the Ad Cre NLS/ CMV recombinant virus.

Although the recombination event should give rise to a replication-defective virus there is always the risk of co-dissemination with replication-competent wild type revertants. Therefore, Ma104 non-permissive cells were infected with the viral stock. The fact that our amplified viral stock did not replicate indicated that no contamination of wild-type virus in the stock.

Moreover, after every round of amplification of the recombinant Adenovirus we checked for the continued presence of the Cre ORF into its genome by PCR using

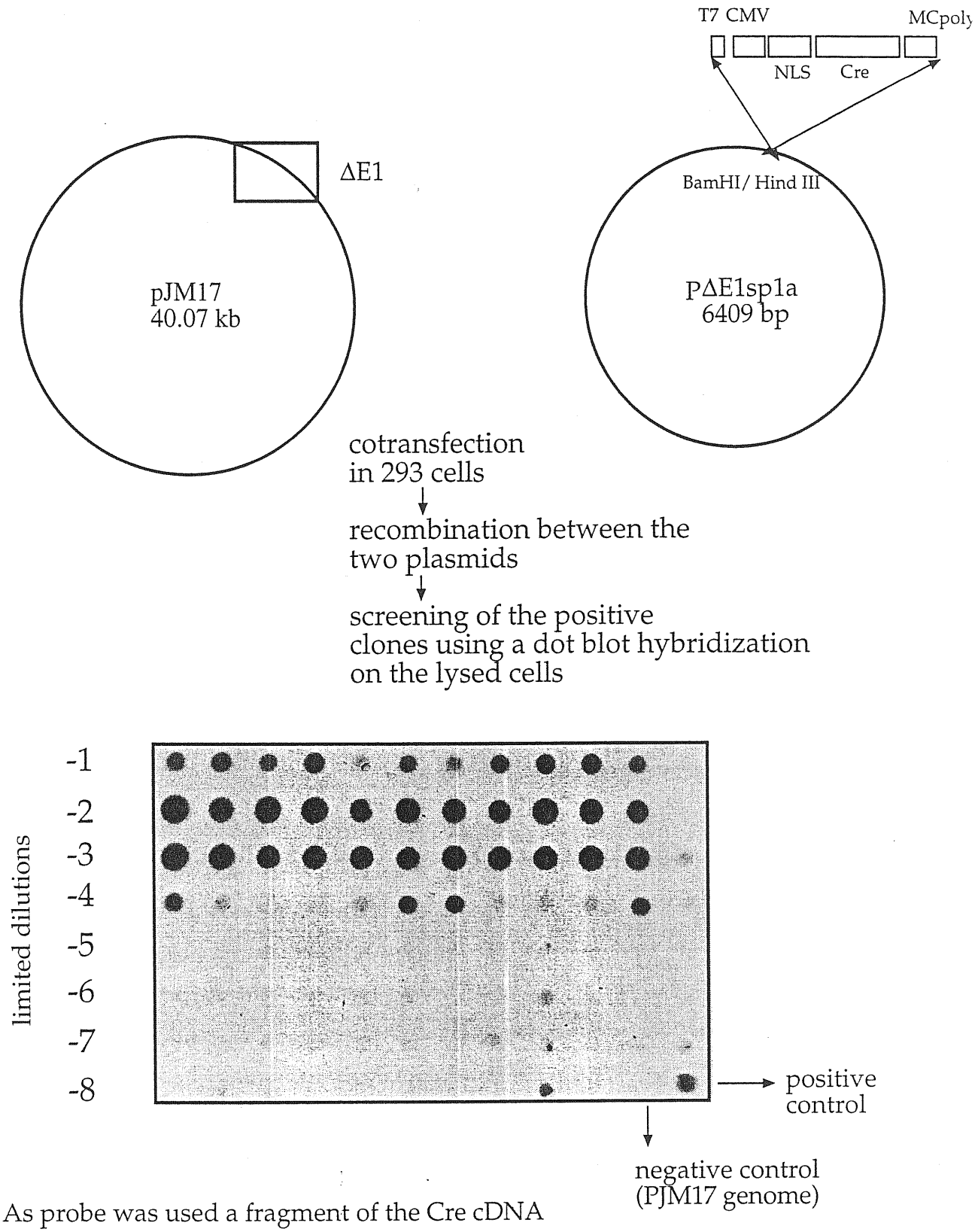


Fig 3.5.2.1: The plasmid pJM17 containing the entire genome of the Ad 5 deleted in the E1 region was cotransfected in 293 cells with schuttle plasmid pΔE1sp1A containinig the Cre protein in order to obtain a recombinant infective Adeno virus able to be used as a Cre protein vector.
The screening of the positive clone was performed using a dot blot approach in which the cellular lysated was diluited in sequential manner in order to allow the isolation of a single positive recombinant clone.
As probe we used a fragment of the Cre cDNA.

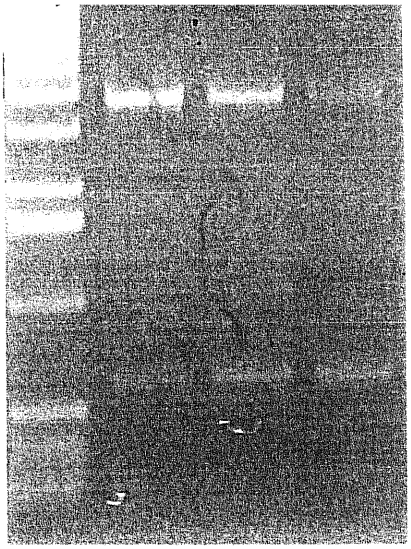
the two primers Cre rec dir 1 (AS) and Cre 775 (S)(the sequence of the primers used is reported in the table 2.1 in the Material and Methods section).

Finally, the defective recombinant Adenovirus containing the Cre protein (Ad Cre NLS/CMV) was tested for its ability to cause the loxP recombination in two type of assays :

a) In the first experiment Embryonic Stem cells stably transfected with a variant of the construct Bls906Corelox/Lac-Z (see section 3.4.1) were infected with different quantities of the recombinant Ad/Cre NLS/CMV virus. After 48 hours the DNA of the cells was extracted and the recombination was tested by PCR. As shown in Fig 3.5.3.1 panel A we used two specific primers which anneal outside of the two loxP sequences. This allows the identification of both the recombined product (the lower band of 600 bp) and of the non-recombined sequence (the upper band of 3900 bp). The lower band was additionally digest with an internal restriction enzyme site to confirm that it was indeed the intended product.

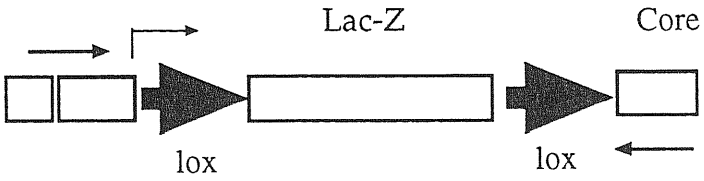
b) In the second assay Hela cells were transfected with a Cat lox/Lac-Z vector (kindly provided by Franco Pagani, ICGEB, Trieste) which is capable of expressing the β-gal protein after recombination. The cells were then infected with the recombinant Ad/Cre virus and after 48 hours were stained as previously described (Fig3.5.3.1 panel B). As shown in this figure, the presence of blue cells confirmed the functionality of our recombinant virus.

An additional experiment was then performed to find which was the minimal percentage of cells infected by the recombinant Ad Cre NLS/CMV that could be detected using PCR. Two 60 mm petri dishes were plated in duplicate with the same number of ES cells stably transfected with the variant of the Bls906Corelox/Lac-Z construct. One of the two petri dish was infected with 60 ul of Ad CMV NLS/Cre ($1x10^8$ viral particle/ml) and after 48 hours the DNA was extracted from both the infected and uninfected dish. The recombined DNA extracted from the first dish was then mixed in different ratios with the non-recombined DNA from the second dish. As shown in Fig3.5.3.2 a ratio of 1/10 between the recombined and non-recombined

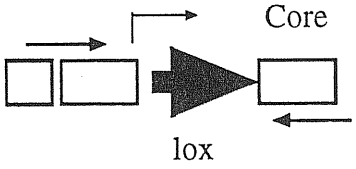


non recombinant product
3900 bp

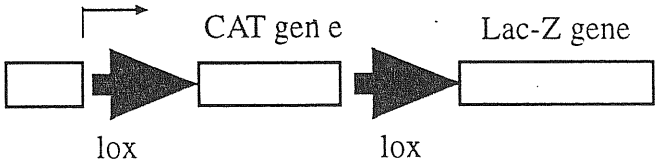
recombinant product
600 bp



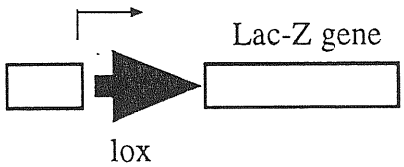
Ad Cre



Fuctionality of the recombinant Ad virus: ES cells stably transfected with a variant of the Bls 906 core lox/Lac-Z construct were infected with adifferent quantity of the amplified recombinant Ad virus used as a vector for the Cre protein.
After 48 hours the cellular DNA was extracted and analysed by PCR. The primers used are localised out of the two lox sequences. This allows to amply at the same moment the recombinant product (600 bp) and the non recombinant product (3900 bp).



AD Cre



Hela cells were transiently transfected with the construct CAT/lox/Lac-Z.
The infection of the cells with the recombinant Ad virus carrying the Cre protein allows the expression of the Lac-Z gene and the staining of the cells.

Fig 3.5.3 .1 : Functionality assays to test the defective recombinant Ad/CMV NLS-Cre virus.

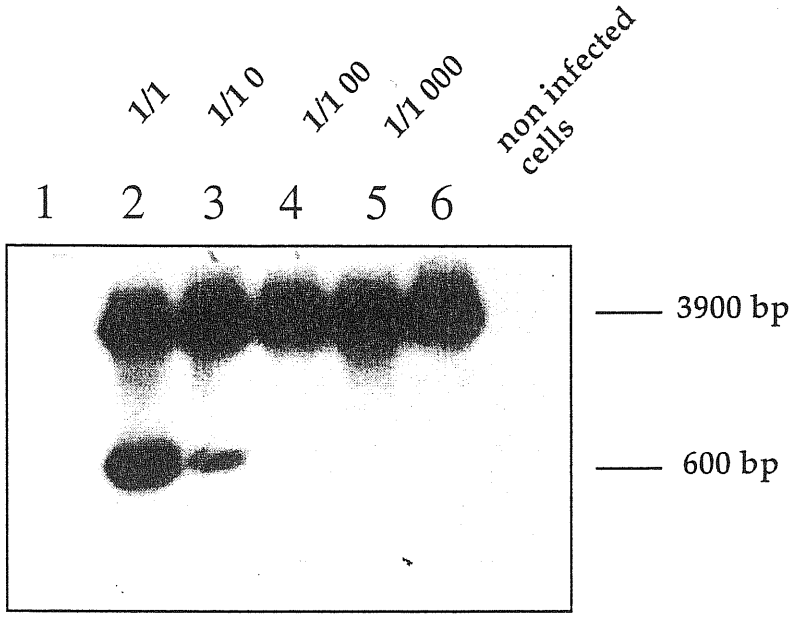


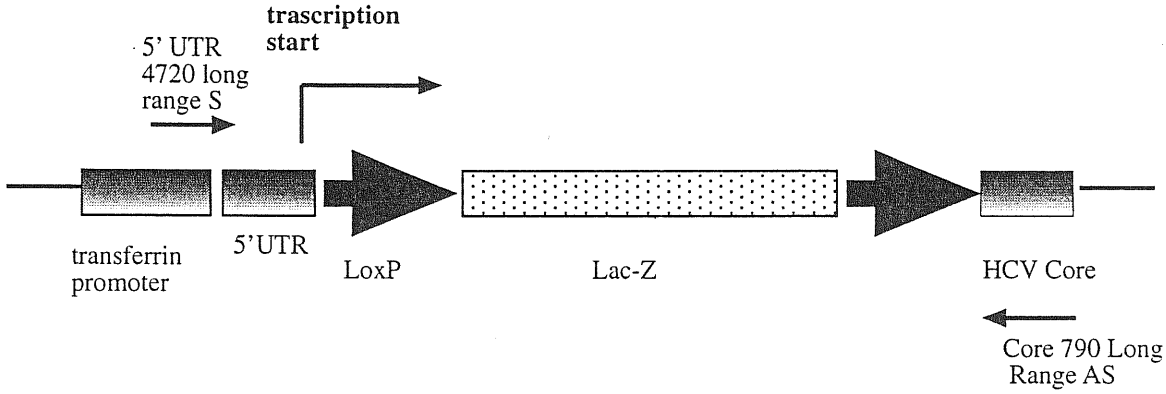
Fig.3.5.3.2: A PCR approach was performed in order to understand what is the minimal percentage of infected cells that can be detected using a PCR.
Two 60 cm petri dish of ES cells stably transfected with a variant of the plasmid Bls906Corelox/Lac-Zwere plated in duplicate. One of the two dish was infected and the after 48 hours the genomic DNA from both the petri was extracted. The recombinant DNA was mixed in different ratio with the non-recombinant DNA that was mantained constant. On this DNA a Long Range PCR was performed as shown in the figure. Lane 2,3,4 and 5 showthe amplified product obtained mixing the DNA from the infected and non-infectedpetri in different ratio.
Lane 6 shows the amplification of the genomic DNA of non infected cells alone and lane 1 the water as negative PCR control.

DNA still allowed to detect recombination lower product. Even assuming that not all the DNA in the first petri dish was successfully recombined by the recombinant Adenovirus from this result was possible to conclude that the presence of at least one infected cells out of 100 can be detected using PCR also taking into consideration the relative PCR efficiency of the 3.9 Kb and 0.6 Kb bands (see section 3.6).

3.6 IN VIVO RECOMBINATION

To test the efficiency of Adenovirus-mediated gene transfer in different tissues in vivo we injected into the tail vein of our transgenic mice $0.5x10^8$ recombinant Ad/Cre viral particle. The mice were 8 weeks old and belonged to three lines (8574, 8576, 8584) which differed from each other by the gene copy number integrated in each genome (Table 3.1). Eight transgenic animals from each line were injected with the recombinant Ad Cre NLS/ CMV virus, two mice were injected with a PBS solution, and two non transgenic mice were injected with the same quantity of the virus. After one week four transgenic animals injected with the virus plus one transgenic animal infected with the PBS solution were sacrificed. In fact, previous work shows that the activity of the Cre protein in a defective Adenovirus system can be already detectable after 5 days after the viral injection in vivo. (Wang et al., 1996).

The DNA was extracted from the liver of the transgenic mice belonging to three different lines (Fig. 3.6.1 lanes 4, 5 and 6) and from a genomic DNA extracted from the liver of a transgenic mice injected with a physiological solution (PBS) (lane 3). In all these experiments the amplification was performed using an Expand Long Template PCR system (Boeringher Mannheim) which is more efficient at amplifying long fragments of DNA (see material and methods). The primers used for this amplification (Core 790 long range AS and 5′ UTR 4720 long range S) were localised outside of the two loxP sequences, and were the same used in the cell culture experiments (see section 3.5.3). Therefore, the PCR analysis should have yielded a 3900 bp product from the intact loxP locus in the transgene or give a 600 bp product resulting from the Cre-induced recombination between the two loxP following



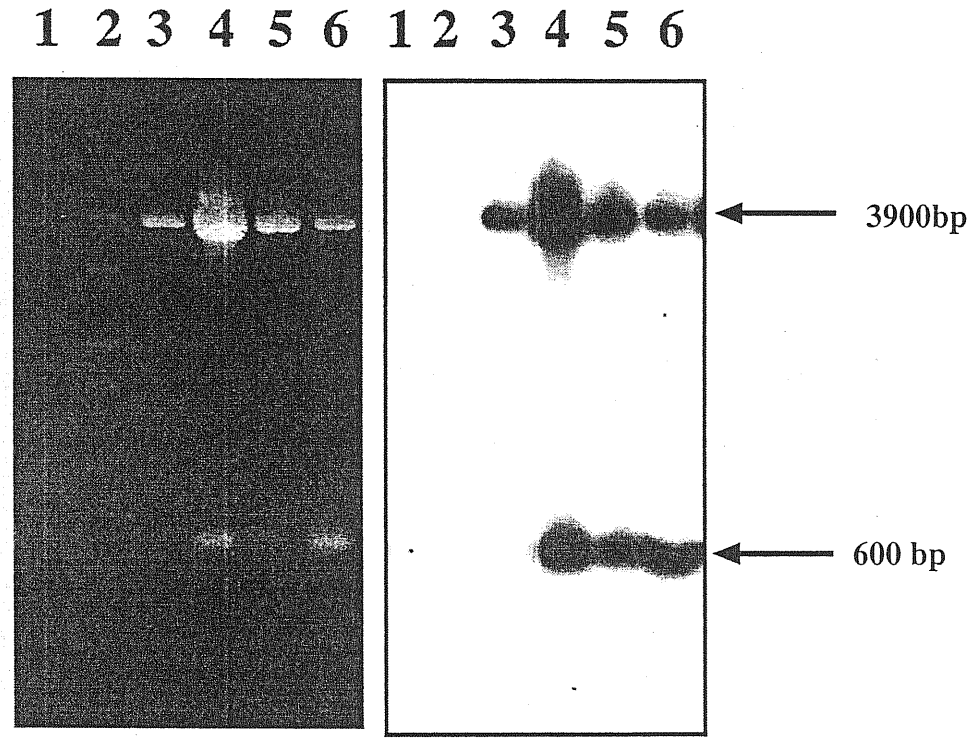


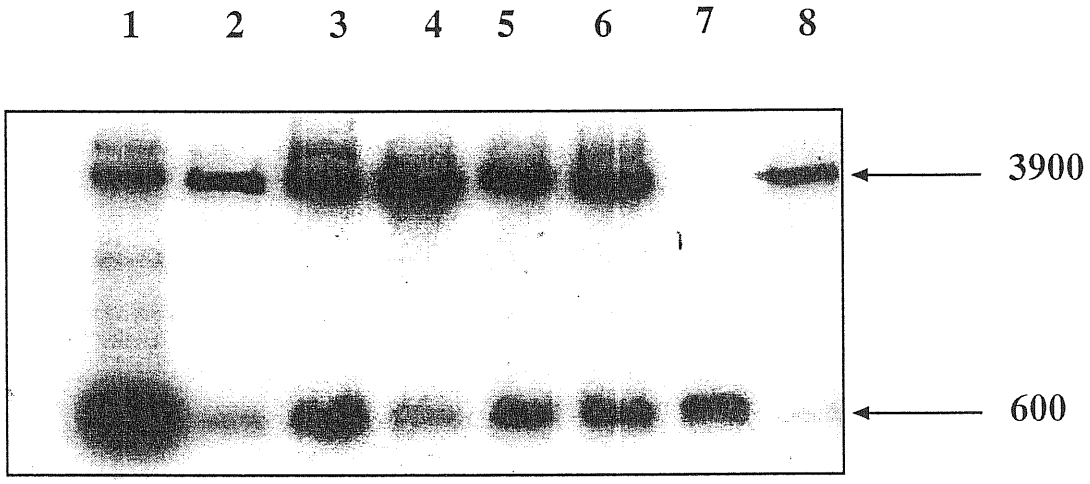
Fig 3.6.1: In vivo recombination observed in genomic DNA extracted from the liver of transgenic mice injected with recombinant Ad/Cre virus. The virus was injected throught the tail vein and the recombination was observed using a PCR approach.
The primers localised out of the two lox-P sequences are shown in the upper panel. Line 1 shows the product of amplification obtained on the DNA extracted from the liver of transgenic animals injected with a physiological solution (PBS) Line 2,3 and 4 show the same type of amplification performed on DNA of three mice injected with the recombinant defective Ad virus. The three transgenic mice belong to three different lines and were all sacrified both after one week from the viral injection.

deletion of the 3.2 kb Lac-Z gene. Fig 3.6.1. shows that recombination was observed in those mice treated with the Ad /Cre recombinant virus while control animals injected with the physiological solution showed no Cre-mediated recombination.

Therefore, eight tissues from each mice were analysed for evidences of recombination using the same long range PCR. As it is possible to note in Fig, 3.6.2. there is a high level of recombination in the liver and this is consistent with the fact that this organ is the prime target for the virus injection in the tail vein. A variable level of recombination was observed in the other tissues analysed: kidney, spleen, lung, heart, pancreas, tail. Only a very low level of recombination was observed in the brain, in agreement with the fact that the brain-blood barrier blocks the viral particles from reaching and infecting the brain tissues. (Doran et al., 1995; Huard et al., 1995; Wang et al., 1996).

Although Fig 3.6.1 and 3.6.2 shows a high level of recombination (approx 50 %) in the liver we have to keep in mind that the efficiency of amplification is much greater for smaller fragments than for larger. To further investigate this we then mixed in the same reaction tube two plasmids: one containing an intact CoreLox/Lac-Z sequence and a second plasmid with the lac Z gene excised. Both plasmids were amplified using the same primers (Core 790 long range AS and 5' UTR 4720 long range S). The results obtained show that the equivalent amplification point of the two fragments results when the ratio between the complete and recombined sequence is 1/10 (Fig 3.6.3 panel A). This means that equivalent DNA intensities of the two fragments can be observed when only 10% of the DNA analysed has undergone a recombination event. This confirms the fact that what we see in the transgenic mice actually represents a much lower level of recombination than what we would have deduced from the simple intensity of the bands.

We also performed a genomic southern blot experiment in order to confirm the in vivo recombination. Using this technique it was not possible to quantify the level of recombination. In fact in agreement with previous evidences reported by Wang et al (Wang et al., 1996)the high copy number integration in our transgene and



In vivo recombination

Fig 3.6.2 PCR analysis of the genomic DNA was performed in order to observe in vivo recombination in different tissues. Sample are from the liver (lane1) pancreas (lane2) kidney(lane3),spleen(lane4),lung(lane5),heart(lane6),tail(lane7) and brain(lane8). The DNA sample were prepared from mice that had received intravenous injection of Ad/Cre virus. The arrows denotes the 3900 b p and the 600 bp recombinant bands. The highest percentage of recombination is observed in the liver. The other tissues show a variable amount of recombination whilst in the brain the extent of recombination is present only in traces.

the random orientation of each copy gene, caused an unpredictable recombination pattern giving rise to a smear in the southern blot. Considering both the efficiency of PCR amplification and the theoretical efficiency of Adeno infection we have calculated that the level of recombination obtained in the liver is approximately 1% .

In order to increase this percentage of recombination we repeat the Ad injection in other mice belonging to the same three lines. The recombinant Ad/Cre virus was injected a second time after a two-day interval. Therefore, each received a double quantity of Adenovirus ($1x10^8$) with respect to the first experiment. It is important to note that the second injection was made two day after the first to avoid any problems that could have been caused by the eventual development of an immunological response against the recombinant Adenovirus. After one week these mice were sacrificed and the level of recombination was determinated both in the liver and in the other tissues. However, no macroscopic increment in the level of recombination was observed in this double injected mice (Fig 3.6.3 panel B).

Finally, to confirm that also in these in vivo experiments the recombined fragment is exactly the product of Cre mediated Lac-Z gene excision the amplified product was cloned in a Bls vectors and sequenced. Its sequence, shown in Fig 3.6.4, indicates very clearly the excision of the Lac-Z gene and the fact that after the ATG of the HCV Core protein one can indeed read the entire ORF of this protein (and the loxP sequence inserted inside).

3.6.1 Persistence of the recombination in the mice liver.

In order clarify whether the Ad/Cre DNA recombination that occurs in the liver of the infected transgenic mice has an influence on the survival of the rearranged hepatocytes we have performed an analysis of the levels of recombinant DNA during an extended period of time. Therefore, over a period of four months, transgenic mice of each of the three lines 8574,8576 and 8584 were sacrificed at different time intervals and the genomic hepatic DNA was subjected to PCR analysis.

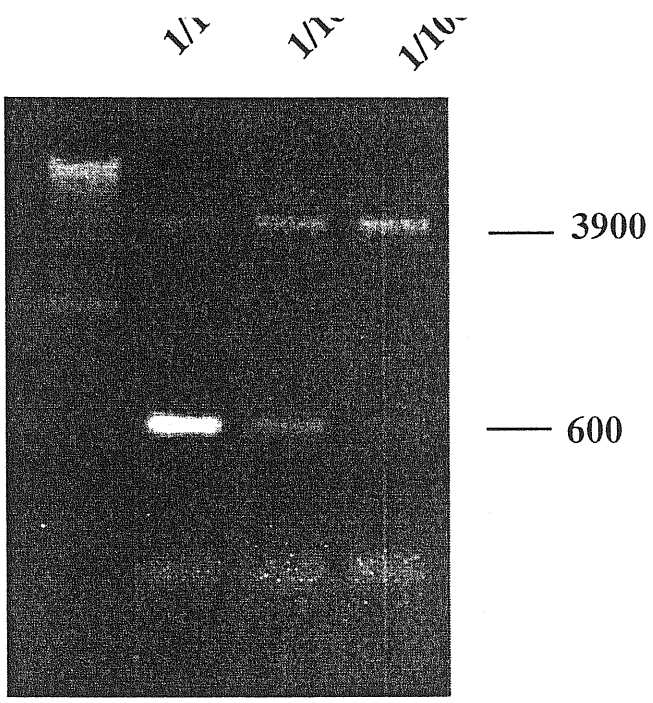


Fig 3.6.3: Panel A shows a PCR system to investigate the efficiency of the amplification. In the same reaction tube different ratios of plasmids containing the whole Bls906Corelox/Lac-Z and a second with the lac Z genl excised were mixed in equimolecolar ratios. Both the plasmids were amplified by the same primers (Core 790 long range and 5' UTR 4720 long range). The results obtained show that the equivalent amplification point of the two fragment results when the ratio between the higher and thes lower band is approximately 1/10.

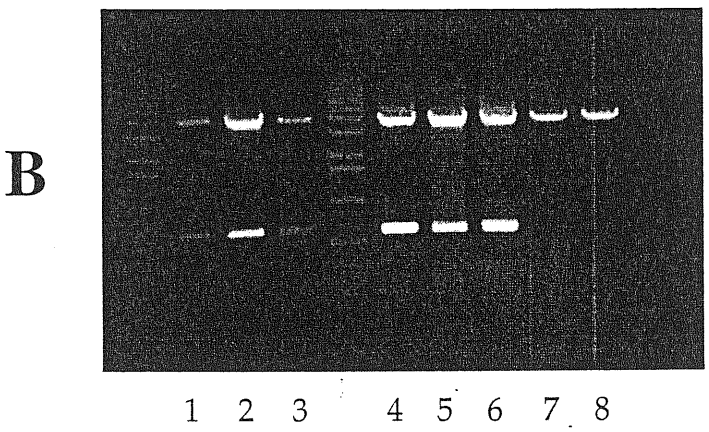


Fig 3.6.3 Panel B shows comparison of the percentage of recombination observed in different mice belonged to the three transgenic line 8574, 8576 and 8584 after a single injection of recombinant Ad/Cre virus (line 1,2,3) and after double injection (line 4,5,6). In the lines 7 and 8 we show the amplification of the genomic DNA extracted from mice injected ones or two time with a saline solution (PBS). No recombination was observed in these two animals.

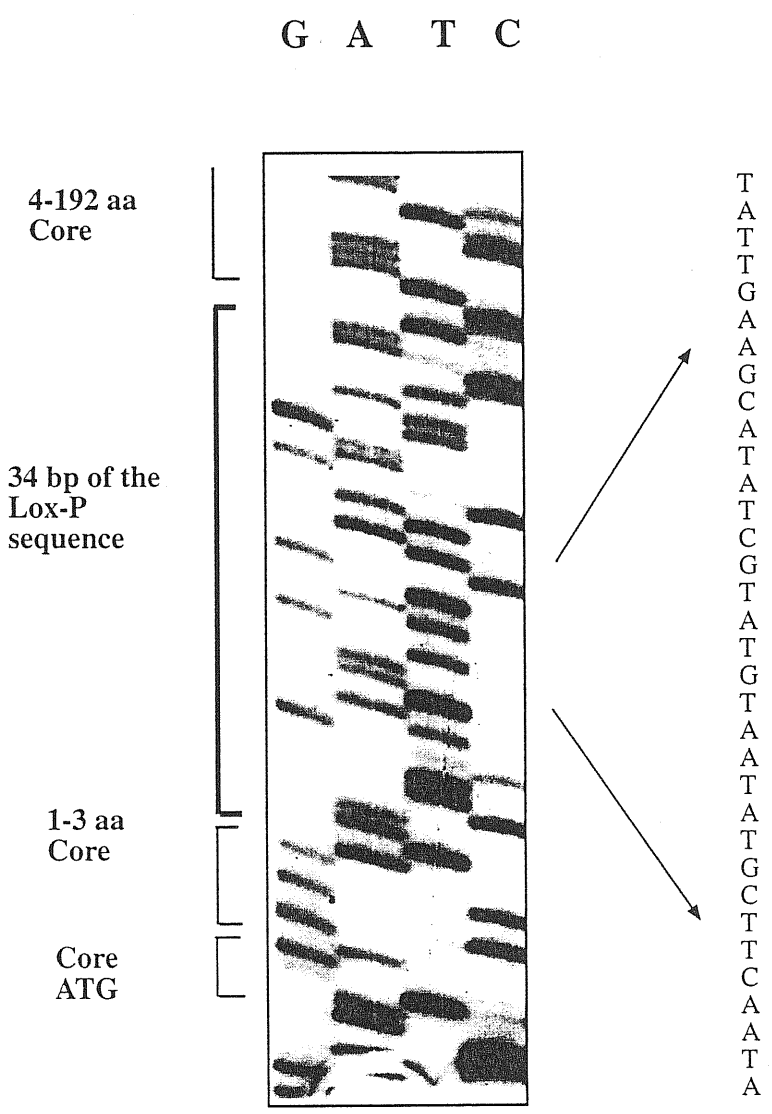
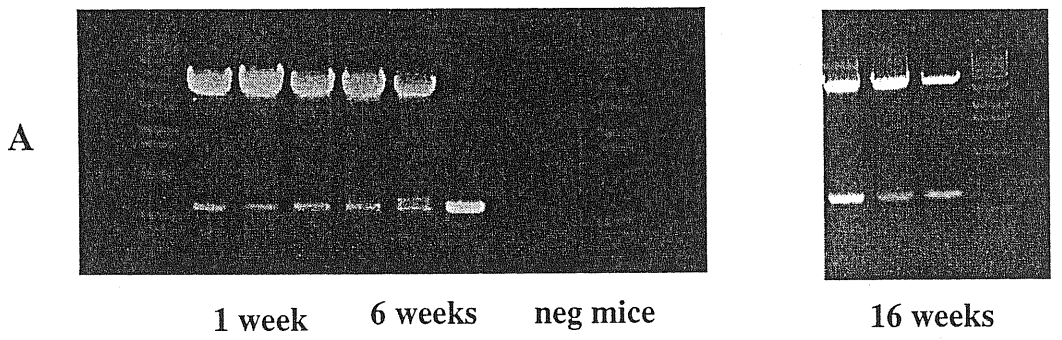
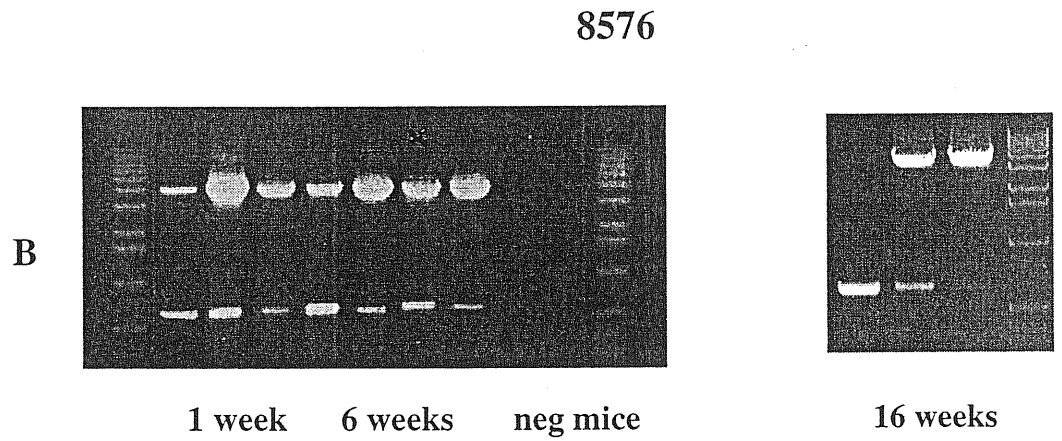


Fig 3.6.4: Sequence of the recombinated fragment of 600 bp obtained in vivo after the Ad/Cre mediated recombination.
As can be seen the sequence of the Lac-Z gene has been excised and the total nucleotide sequence of the HCV Core proteinand only insertion one 34 bp Lox-P sequence has remained.





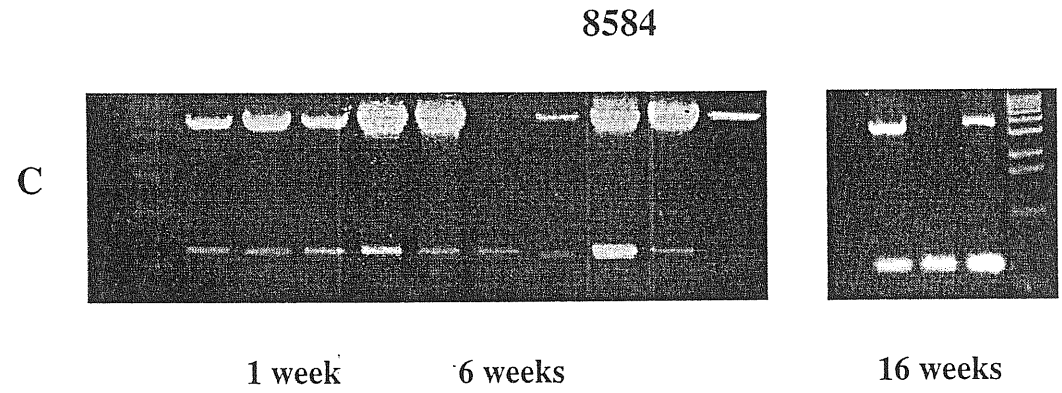


Fig 3.6.5: The turnover of the infected hepatoytes in the liver of the injected transgenic mice was followed by testing the level of Cre mediated recombination over a period of time.
In the three different panels A,B and C the DNA extracted from the liver of the mice belonging to three different lines was amplified in order to investigate any changes in the level of recombination occur.

As shown in Fig 3.6.5 no significative variation in the recombination level of each transgenic lines could be observed for at least 16 weeks. It should be noted that there is a high variability in the percentage of starting recombination observed in the different animals, probably a consequence of the efficiency of the tail vain injection.

In addition the amplification of only the lower band in three samples (see fig. 3.6.5)was observed to be due to a hinibition effect on the Expand Long Range Taq polymerase by the DNA extracted from the liver of these three injected mice. In fact when this DNA was mixed with DNA which had yielded in previous PCR amplification both the upper and the lower bands, only the lower band could be detected. Therefore, the absence in these samples of the upper (un-recombine) band must be considered an artefact and does not imply that on these sample a total recombination had occurred.

3.6.2 Persistence of Ad/ CMV NLS-Cre in the liver of the injected transgenic animals.

To test the persistence of the recombinant Adeno/Cre genome in the liver of the infected transgenic mice, we performed a genomic southern blot on these animals belonging to the three lines 8574, 8576 and 8584.

Total DNA was prepared from liver of transgenic animals at 1, 6 and 16 weeks after viral injection. Upon HindIII/ClaI digestion the Adeno/Cre virus genome yields a 1.6 Kb fragment harbouring a fragment of the Cre recombinase gene. This fragment was detected using as a probe a fragment of the Cre gene. As shown in the Fig. 3.6.6 it shows that is possible detect the presence of the episomal Adeno/Cre virus only after one week to the infection in all three lines (lane 1,4,7) whereas the genome of the recombinant adenovirus was lost already after six weeks (lane 2,3,5,6,8,9).In addition, the mice belonging to the same lines analysed before the injection of the adeno/Cre virus didn't show any positive signal (lane 10,11,12).

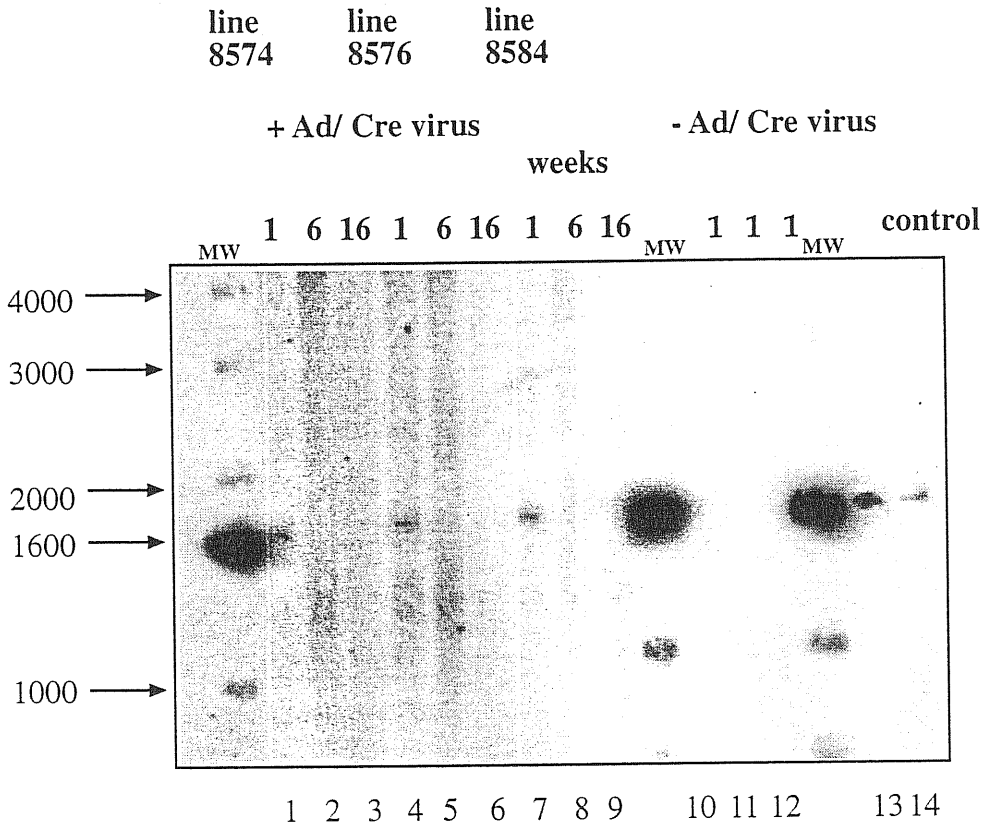


Fig. 3.6.6: genomic southern blot of the liver of an Ad/Cre injected mice after 1 (lane1, 4, 7) 6 (lane 2, 5, 8) and 16 weeks (lane 3, 6, 9) after the Ad infection was performed to evidentiate the presence of the Adeno/Cre genome over a long period of time. The genomic DNA was digested with two enzymes which cut in the Adeno/Cre sequence and the presence of the viral DNA was detected using as probe a fragment of the Cre ORF.
The genomic DNA of the same mice sacrified after 1 week post injection of a saline solution (PBS) were extracted and digested with the same enzymes. In this case a positive signal was not observed (lane 10,11,12). As control we used the plasmid pΔE1sp1A (lane 13 and 14) .

3.6.3 Search for antibody against the Core protein

The presence of a significant recombination in the liver of transgenic mice should have resulted in the production of HCV Core protein and perhaps in a subsequent immunological answer against it. To investigate this possibility we bled the infected transgenic mice before the viral injection in order to have a preimmune sera and then at regular intervals over a period of four month after the Ad/Cre injection.

Two types of experiments were performed in order to identify the presence of antibodies against the HCV Core protein.

3.6.3 .1 ELISA assay

Three overlapping synthetic peptides covering residues 1 to 59 (Pep C1:residues 1-20, pep C-2:resides 21 to 41 and pep C3:residues 27 to 59) were used to test the sera from all infected transgenic mice. This corresponding to the most important immunological region of the HCV Core protein . No significant results against these peptides was recovered over a long period of time. Table 3.6 reports the mean value obtained from 8 injected mice and three control (non injected) mice after each bleeding .

As synthetic peptides can be quite inefficient at detecting immunological responses, especially against conformational epitopes the same experiments was performed using a set of recombinant proteins displaying on their surface three well known immunoreactive HCV Core regions: 1-20 (C1), 21-40 (C2) and 32 to 46(C3). The better ability of these proteins to detect antibodies against these sequences as compared to that displayed by synthetic peptides had already been demonstrated (Buratti et al., 1997). We then tested the sera obtained from all the mice at different time intervals after the injection of the recombinant Ad virus. Unfortunately since a preimmune reactivity against the C3 protein was often detected the sera were tested for reactivity only against the C1 and C2 regions . However, none of the sera showed any significant reactivity against either protein .

Line 8574

injected mice

preimmune	0.169	0.190	0.153
16 weeks	0.200	0.172	0.162
24 weeks	0.321	0.167	0.174

Cap 1 (aa1-20) Cap2 (aa 21-41) B12F8 (aa 27-59)

non injected mice

preimmune	0.150	0.200	0.170
16 weeks	0.170	0.200	0.180
24 weeks	0.153	0.280	0.250

Cap 1 (aa1-20) Cap2 (aa 21-41) B12F8 (aa 27-59)

Line 8576

injected mice

preimmune	0.167	0.160	0.173
16 weeks	0.175	0.152	0.162
24 weeks	0.200	0.195	0.163

Cap 1 (aa 1-20) Cap2 (aa 21-41) B12F8 (aa 27-59)

non injected mice

preimmune	0.160	0.200	0.150
16 weeks	0.170	0.165	0.170
24 weeks	0.163	0.149	0.145

Cap 1 (aa 1-20) Cap2 (aa 21-41) B12F8 (aa 27-59)

Line 8584

injected mice

preimmune	0.161	0.171	0.145
16 weeks	0.159	0.159	0.181
24 weeks	0.180	0.182	0.174
	Cap 1 (aa 1-20)	Cap2 (aa 21-41)	B12F8 (aa 27-59)

non injected mice

preimmune	0.174	0.164	0.170
16 weeks	0.150	0.155	0.191
24 weeks	0.143	0.124	0.110
	Cap 1 (aa 1-20)	Cap2 (aa 21-41)	B12F8 (aa 27-59)

Table 3.6: in this table is reported the mean value obtained from the ELISA experiments in all the three injected lines. Three overlapping synthetic peptides covering residues 1 to 59 (Pep C1:residues 1-20, pep C-2:resides 21 to 41 and pep C3:residues 27 to 59) were used to test for the presence of specific anti Core antibodies over a period of 6 months.

3.6.3.2 Western blot screening of the mice sera.

We could still not rule out the possibility an eventual antibody response developed in the injected mice could have been directed against other regions of the HCV Core protein beside its N-terminal portion.

Therefore, we cotransfected in the NMuli cell line the plasmid Bls906Corelox/Lac-Z and the plasmid pΔE1sp1A Cre NLS/CMV to express the entire HCV Core protein (including the lox P sequences). In order to increase the efficiency of HCV Core protein expression we used a Vaccinia virus system

described in the section 2.2.8.3 to activate the transcription instead of relying on the transferrin promoter. After 24 hours the cells were harvested and the cellular lysate was loaded on a polyacrylamide gel in several lanes. We then performed a western blot (as described in Material and Methods) loading an equal amount of cellular extract in each lane. The membrane was subsequently cut in different strips and each was used to test the animal sera at a 1/10 dilution in PBS for the presence specific anti-core antibodies. As positive control one of the strip was incubated with the monoclonal Antibody B12.F8. However, Fig..3.6.7. shows that no specific signal was visible in correspondence to the molecular weight of the Core protein.

3.6.4 Expression of the Core protein in the liver of the transgenic mice

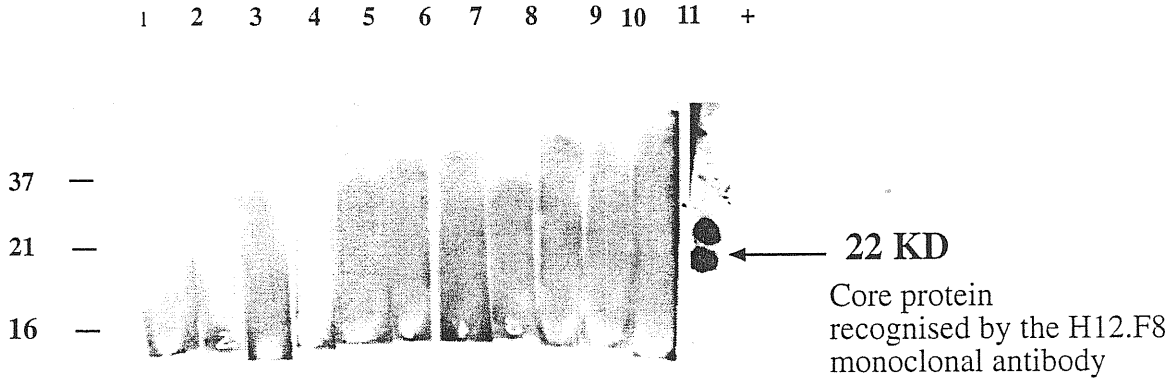
Concomitantly with the experiments described above we also performed other experiments to attempt the direct identification of the Core protein in the liver of the Ad/Cre infected mice. This was initially attempted by performing a western blot of a total liver protein extract from each animal.

3.6.4.1 Western blot on the total transgenic animal liver extract.

The total liver proteins were extracted from the mice as described in Material and methods, run on a 15% SDS-PAGE polyacrylamide gel and blotted.The presence of the HCV Core protein was then tested using the human monoclonal antibody B12.F8 in different concentrations but no positive signal could be detected even after developing of the western blots using the sensitive ECL technique. In order to increase the quantity of the extract analysed the proteins obtained from the homogenised liver were also immunoprecipitated using B12.F8 and blotted later. Also in this case the use of the monoclonal B12.F8 anti Core antibody did not reveal a positive signal .

3.6.4 2 Immunohistochemistry

LINE 8584



LINE 8574

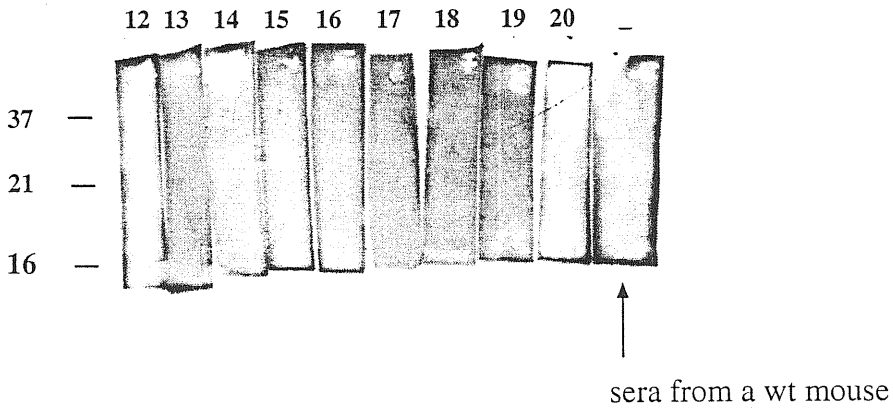


Fig 3.6.7 The sera of 20 transgenic mice belonging to the 8584 line and to the line 8574 were tested after 16 weeks to the Ad/Cre injection for their capability to recognise the HCV Core protein expressed in a cell culture system..
In the lane of the positive control (+) the Core protein is recognised by the monoclonal human antibody B12.F8 in a dilution of 1/100. As negative control we have used the sera of a wt mice .
The sera of the transgenic mice tested at a concentration of 1/10 show only a level of aspecific background.
The same result were observed also for the line 8576.

Finally, we also tried to detect the presence of HCV Core protein in the mice liver tissue using an immunohistochemical approach (described in material and methods). High-titre, anti HCV positive sera of chronically infected patients was used as a source of primary antibodies in the histochemical experiments. These sera were tested first in western blot as shown in Fig 3.6.8 which confirmed that its reactivity against the HCV Core protein obtained after recombination in western blot was comparable with that obtained using the human monoclonal antibody B12.F8 and that no other aspecific bands were observed in the same experiment. This sera was then used in immunohistochemisty experiments on paraformaldehyde-fixed and paraffin-embedded sections of transgenic mice livers. Fig 3.6.8 shows that despite the positive result observed on paraformaldhyde-fixed paraffin-embedded sections obtained from a human biopsy no imunohistochemical evidence of HCV Core protein expression in the liver of the transgenic mice could be detected.

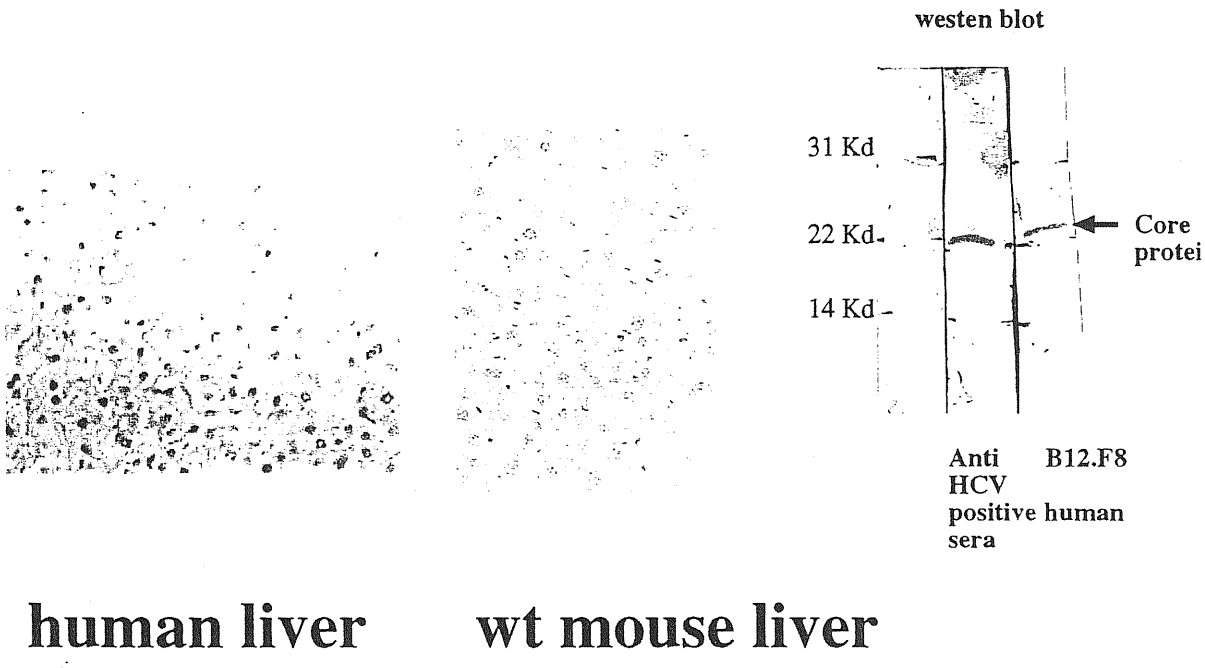


Fig 3.6.8: High-titre, anti HCV positive sera of chronically infected patients was used as source of primary antibodies in the immunohistochemical experiments. The sera was tested first in western blot to compare its reactivity with that obtained using the human monoclonal antibody B12.F8.
Despite the positive results observed on paraformaldhyde-fixed paraffin embedded sections from a human biopsy no evidence of HCV Core protein expression in the liver of the transgenic mice could be detected.

Chapter 4

Discussion

HCV infections are a major cause of morbidity and mortality worldwide (Hsia and Seeff, 1992; Lemon and Brown, 1994).The virus was identified through molecular cloning of the HCV genome, which has facilitated the understanding of its structure and epidemiology (Choo et al., 1989; Houghton et al., 1994; Omata and Kato, 1994)

Despite recent advances the mechanism of hepatocellular injury in chronic HCV infection have not yet been fully delineated. Infection with HCV results in both humoral and cellular immune response, although the magnitude and the specificity of these responses appear to be quite variable among infected individuals (Shimizu et al., 1994). There is a growing body of evidence indicating that the immune mechanism is involved in the cellular damage caused by viral infection since humoral response has been detected against all of the viral antigens. However, the adverse role possibly played by either antibodies or by cytotoxic lymphocytes in the pathogenesis of HCV infection remains to be investigated. Therefore it is relevant to determine whether expression of viral proteins leads to direct liver injury independently of the host immune response . For this reason a lot of efforts have been focused last years in the production of an animal model able to shed light on the directly-acting viral pathogenic activity.

The first steps aimed at clarifying these issues were to express of the HCV viral proteins in various tissue culture systems (Dobuisson et al., 1994; Spaete et al., 1992) and, more recently, the in vivo transfection of HCV cDNA into rat liver has been reported (Takehara et al., 1995)

Unfortunately, the absence of an efficient cell culture system and of a small animal model that can be readily infected with HCV makes any "in vivo approach"

very difficult. Nonetheless, advances in the field of transgenic technology still offer the best opportunity to develop valuable models for HCV human disease.

The expression of the HCV Core protein (Moriya et al., 1997; Pasquinelli et al., 1997) and of the structural proteins of the virus (E1 and E2) (Kawamura et al., 1997; Koike et al., 1995) in the liver of transgenic mice have contributed pivotal information on the cytotoxicity of these protein during HCV infection. The evidences show that despite a good expression level, and a correct processing these structural proteins were not directly cytopathic in these animal models. However, all these models were unsuited to study any pathogenic effects of the immune responses against the expressed viral product because transgenic mice expressing these antigens in utero were tolerant to the antigen. Therefore, obtaining a controlled viral gene expression in an animal model could clarify the role played by the immune system in HCV virus infection.

We have developed a model to obtain a controlled expression of the HCV Core protein and to obtain this we used the Cre-loxP recombinase system from bacteriophage P1. Our efforts were focused on the aim of obtaining a transgenic animal model with the following characteristics:

a) Zero expression of the HCV Core protein before an Adeno/Cre infection

b) Moderate to low expression of the HCV Core protein after Cre induced recombination

c) Infection only on a fraction of hepatocytes because the infection of the entire liver could be lethal for the mice

d) Persistence of the rearranged hepatocytes

e) Immune reaction to the HCV Core protein

The Cre recombinase system has already been extensively used both in cell culture and in transgenic animals to induce site specific DNA recombination leading to gene activation or deletion(Gu et al., 1994; Kuhn et al., 1995; Lakso et al., 1992; Orban et al., 1992; Sauer and Henderson, 1989) .

When we started this work the use of a recombinant Adenovirus harbouring the Cre recombinase to rearrange a "floxed" gene (the gene contained between the two loxP sequences) in a cell colture system has been just suggested by Anton 1995(Anton and Graham, 1995). Later studies by Wang et al (Wang et al., 1996) showed that a Ad/Cre virus is capable of rearranging a loxP-tagged transgene to a variable degree in several mice tissues and more recently, Rohlmann et al (Rohlmann et al., 1996)extended these observations and demonstrated the feasibility of inducing temporally controlled somatic gene disruption in parenchyma liver cells by Adenovirus-mediated transfer of Cre recombinase. In addition, the Adenoviral Cre/loxP system has also been shown to possess an advantage over conventional transgenic approach, that of avoiding the requirement of the mating of two transgenic lines, the first necessary for the expression of the selected transgene and the second for the Cre gene (Akagi et al., 1997; Wang et al., 1996). Additionally, since the recombinant Adenovirus is normally not integrated, this method does not further modify the genome. Finally, the ability of this virus to infect a broad range of cells types across many species (Brody and Crystal, 1994) allows this vector to be suitable for the analysis of gene function in the development of disease.

In our project, we designed an expression cassette in which the HCV Core protein cDNA was separated from the liver specific promoter plus the HCV 5' UTR by a spacer region flanked by loxP sites. This spacer would prevent the HCV Core protein expression unless the spacer was excised.

The tissue specific expression of the HCV Core protein in mice liver was obtained using the liver specific region of the murine transferrin promoter (-581 and +50 bp). In fact, the use of this promoter fragment in transgenic mice (Idzerda et al., 1989) have confirmed its functionality and tissue specificity in vivo.

A fusion gene containing the -581 to +50 bp fragment of the murine transferrin promoter linked to the Lac-Z codified region was used to validate the functionality of this tissue specific promoter (chapter 3.3.1). Functionality transfection assays were performed in different cellular lines and allowed us to conclude that the -581 to +50

bp fragment of the murine transferrin promoter was able to drive the Lac-Z gene expression in liver cells and more precisely that it was more efficient in mouse cells when compared with human cells. Since this fragment of the mouse transferrin promoter is highly homologous with the human promoter, the higher expression of the Lac-Z reporter gene in the liver murine cell line (NMuli) is probably due to the presence of specie-specific factors present in these cells. Alternatively, another explanation of the different levels of Lac-Z gene expression can also be due to the different characteristic of the two cellular lines. In this sense, partial differentiation may cause the absence of some factors whose function is to ensure maximal transcription of a tissue specific gene. For example, in hepatoma cell lines differentiation is associated with the loss of the factor APF required for transcription of the albumin gene (Cereghini et al., 1988).

In our experiments the efficiency of the mouse transferrin promoter fragment was low in comparison with the efficiency of the CMV promoter used as a positive control in all the experiments. This does not necessarily represent an undesirable characteristic because a strong liver specific transcriptional promoter is not necessarily adequate for establishing the transgenic mice we wished to develop . In fact, high-level expression may be lethal to the mouse foetus or toxic to the adult tissue.

Several in vitro and in vivo studies have confirmed that a transient Cre protein expression is sufficient for the event of recombination to take place (Araki et al., 1995; Sakai et al., 1995). We confirmed the feasibility of the Cre-loxP system using high-yield Vaccinia-virus transient cotransfection system in Hela cells that following the recombination event resulted in expression of the HCV Core protein. The result show in chapter 3.3.3 indicated both efficient recombination and production of Core protein only in the presence of the Cre recombinase protein. In fact, the activity of four independent Cre clones was tested and these experiment allowed us to select the functional Cre clones use later for the production of the recombinant defective Adeno/Cre .

We then tested the efficiency of the transferrin promoter to drive the expression of the HCV Core protein after the recombination event: vector Bls 906 Corelox/lac-Z containing the two lox-P sequences and the shuttle vector PΔE1sp1A containing the entire ORF of the Cre protein plus a nuclear signal, were cotranfected in the mouse NMuli cells. The results obtained in these experiments (chapter 3.3.3) show a low level of expression of the transferrin promoter in comparison with the efficiency of the T7 promoter in a Vaccinia virus system. The difference in the amount of the HCV Core protein expressed after recombination is due exclusively to the different efficiency of the two promoters, keeping in mind that the Vaccinia virus system is able to increase the efficiency of the T7 promoter al least ten times in a transient transfection system. In this experiment the same type of cotransfection was performed and the number of transfected cells was strictly controlled. Nonetheless, using the transferrin promoter a detectable HCV Core protein was expressed, confirming again the viability of this liver specific fragment of the transferrin promoter in an hepatic cellular system . Finally, since the presence of the HCV Core protein was detected using a human monoclonal antibody (B12.F8) against a conformational epitope (the N-terminal region of the Core protein) this confirmed that the introduction in this region of 11 aminoacids doesn't change the conformational structure of this protein.

These first results confirmed the expectation to have a system in which the moderate activity of the trasferrin promoter drive HCV Core protein expression only in the presence of the Cre recombinase protein.

Although there was an Adeno Cre described in literature (Anton and Graham, 1995) this was not available and we had to created an Ad/Cre replication-deficient recombinants virus. This virus can be grown without helper virus on trans-complementing 293 cells (Berkner, 1988; Graham et al., 1977).The title of the produced recombinant adenovirus stock resulted to be $1x10^8$ viral particle/ml using a limited dilution assay . In vitro experiments described in chapters 3.5 showed that Embryonic Stem cells stably transfected with a variant of the construct

Bls906Corelox/Lac-Z (the 5′ UTR of the HCV genotype 1 is replace with the 5′ UTR of the genotype 3) were subjected to recombination of approximately 1% when infected with 40 µl of the adeno viral stock (the efficiency of Ad infection and the efficiency of PCR amplification was taken in consideration in the calculation of this value). In addition ,recent studies have confirmed that the tail vein viral injection of the virus in the mice allows a strong transient expression of the exogenous gene in the liver (which seems to be the primary target of this peripheral intravenous injection) (Herz and Gerard, 1993; Stratford-Perricaudet et al., 1992) In agreement with these results, in our transgenic mice the injection of the a recombinant Ad/Cre virus into their tail vein caused a variable level of recombination in the different tissues analysed. Our data confirm the results shown previously by Wang et al (Wang et al., 1996) where the higher level of recombination was observed in the liver. Interestingly, our results also showed that recombination was practically absent in the brain confirming previous observation that the virus is unable to pass the brain blood barrier .

A recombination comparable to the one obtained in vitro was observed in the mice liver when 500 µl of the stock virus were injected into their tail vein. It is also important keep in mind that the a certain variability in the level of recombination can be observed. This variability is not due to a different infectivity of the virus used for the injection in transgenic mice because in all the experiments we used the same viral stock. Therefore, it is likely caused by the variability of the viral injection methodology in the mice. In any case the comparable level of recombination observed both in vitro and in vivo (1%) suggest that also in the animals there is a viable and efficient Cre mediated recombination in vivo. On the other hand, this percentage of recombination ensured that not all the hepatic cells were infected with the virus, and this was in agreement with our desires since the infection of the entire liver could be dangerous for the mice. Our data confirms that Ad vector can be used effectively to achieve efficient Cre/lox recombination in vivo (Akagi et al., 1997).

Since it was already reported that the level of recombination Ad/Cre mediated can be increased in a transgenic system using a higher dose of virus injected (Bout et al., 1994) we have tried to do this by performing a two viral injections after a two day interval. However, the non quantitative PCR assay used to detect the level of recombination in vivo after one or two viral injection did not evidentiate any detectable increase in the percentage of recombination after the double injection. In any case the animals injected with a double dose of recombinant Adenovirus were used for all the successive assays. Nonetheless, the moderate level of recombination(1%) in this system was expected to be sufficient to allow a detectable expression of HCV Core protein.

A timing course study on the Ad/Cre mediated recombination in the mice liver showed that the level of DNA recombination remained around the same value (1%) for at least 16 weeks. On the other hand, the experiments of southern blot performed to identify the presence of the episomal Adeno/Cre DNA over a period of time, show that the viral genome is present after one week to the infection, but was lost completely after six weeks. Regarding the persistence of the Adenovirus genome in the infected cells, a lot of controversial still exists (see below). In fact, the deletion of the Ad E1 gene region results in a failure of the replication-deficient Ad vector to activate both early and late phase transcription from the viral genome, and consequently expression only of a transgene under the control of a constitutive or specifically active promoter is achieved in the target cell. While this genetic barrier to adenovirus gene expression is extremely efficient, it is not absolute. Even limited breakthrough to Ad gene expression could be problematical and in this sense the virus could by itself affect the physiology of the target cell or induce a cellular immune response (Dedieu et al., 1997; Engelhardt et al., 1993; Engelhardt et al., 1994). There is some evidence showing that as the injection of a recombinant Adenovirus in vivo elicited MHC class- I-restricted CD+8cytotoxic T lymphocyte response leading to loss of the exogenous gene expression by mechanisms that include destruction of virus-infected cells.(Wang et al., 1996; Yang et al., 1994; Yang et al., 1996; Yang et al.,

1995; Yang et al., 1994; Yang et al., 1996; Yang et al., 1995)In addition, there are evidences in literature conforms a great variability in the persistence and expression of a gene transferred into liver using a Ad vector. A long time presence of the recombinant Ad virus has been observed in several examples and it has been suggested that yet unidentified genetic loci influence the persistence of adenovirus-mediated hepatic gene expression in vivo and that these effects are mediated at least in part, by the antigen specific immune system.(Dedieu et al., 1997).

Our data show a level of genomic recombination in the transgenic hepatocytes of approximately 1% after the injection of recombinant Adenovirus. The persistence of this level of recombination for long time and the loss of the Adeno/Cre DNA early after the injection suggests:

a) absence of a rapid turnover of the hepatocytes due to a liver damage

b) absence of a cellular immunity against the Adeno viral proteins with following disruption of the infected and consequently loss of the recombinant cells. In agreement with the work of Akagi et al (1997) the loss of the episomal Ad/Cre recombinant virus is thought to be the result of its dilution . In our case, this situation is favoured by the not extreme high viral dose injected in our mice, which excludes a possible breakthrough of low-level expression of the Ad genome that could be observed following the use of a high input multiplicity of infection.

Our observations are also in keeping with these of Akagi et al (Akagi et al., 1997) in which the authors observed a high level of Ad/Cre recombination that remained practically constant for at least 60 days. However, this does not imply that the shuttled gene is always viable. In fact, during this time the expression of the "floxed" gene decreased dramatically . The authors explained this discrepancy by considering that the "floxed" transgene is not expressed in all the infected cells but that the decrease in the transgene expression of the blue cells was due to the immunogenic reaction against the immunodominant exogenous protein rather the viral protein.

Contrary to the positive results that we obtained in the experiments of northern blot and RT-PCR in a cellular system the same experiments performed on the transgenic animals did not detect a transcribed transgenic mRNA. In addition we were not able to detect the presence of antibodies against the HCV Core protein over a period of 4 months following the Ad/Cre injection despite persistence of recombination. In fact, the constant DNA recombination observed for the entire period is in agreement with the situation in which the in vivo Ad/ Cre injection doesn't cause neither an immunological answer against the endogenous HCV Core protein nor a reaction against the viral proteins. In addition , the recombination rate detected by PCR (see Fig 3.6.1 and Fig 3.6.5) may not give a direct indication of how many cells will produce Core protein. In fact, most of the recombination could result in an improductive configuration, particularly in the situation were 10 to 50 gene are integrated in cluster (not necessary in the some orientation). To test this hypothesis and be certain that there are no single cells producing HCV Core protein we used a very sensible technique such as immunohistochemistry were a single expressing cell will be viable. Despite the functionality of the antibody used in this technique no positive HCV Core cells were observed in the liver of injected transgenic mice.

Many hypothesis can be formulated to explain this negative result. It is important to keep in mind the much higher complexity of an in vivo system in comparison with the more easy functionality of a cellular transient system. There are several examples which suggest that results from transient transfections must be treated with caution and may not always yield an accurate picture of a stably transfected system or transgenic animals. In fact, certain enhancer elements may not function unless integrated into an appropriate chromosomal structure (Tapscott et al., 1992) and elements that appear essential in transient assay may not be required in transgenic animals(Swift et al., 1989). In addition, DNA incorporated into a mouse genome is integrated randomly and in a unpredictable copy number. It is known that the integration site often has a profound effect on the expression of the transgene since the expression of the transgene reflects in part the general features of

the chromosomal region into which it is inserted. Recently, Bronson et al (Bronson et al., 1996) proposed a homologous recombination approach to insert a single copy of the transgene in a selectable chromosomal locus of a costitutively open chromatin region. In this situation tissue and developmentally specific promoter and enhances can be expected to retain their natural specificity when used to drive transgene expression. Therefore, integration of the transgene in a single copy number and in a well defined chromosomal region avoids many of the problems associated with randomly inserted transgene.

In our case, the transgene was not transcribed in all the 12 transgenic lines obtained. Although this result could be expected in the case of one line in which the transgene was integrated in the Y chromosome , it was very unlikely that the random transgene integration had always occurred in a heterochromatin region in all the other 11 transgenic lines.

Therefore, an additional hypothesis that could be proposed to explain the absence of HCV Core RNA transcription could be alternatively found in the role carried out by the methylation in the genes transcription regulation. Regarding this, in the work published by Kato et al (Kato et al., 1996) the authors found that despite the good result obtained in a transient expression system, it was impossible to find HCV-specific RNA transcript in the transgenic mice produced. In this work the authors proved that methylation of HCV cDNA was the cause for its inactive expression in transgenic mice and that this phenomenon might have occurred in other stable systems for expression of the HCV genome.

Finally, the absence of a specific RNA transcript in all the transgenic lines in vivo may be caused by the type of construct used. In fact, all the models of transgenic mice proposed to investigate the mechanism of HCV infection are all characterised by the absence of the HCV 5' UTR region in their design. In the model of Pasquinelli et al (1997), Kawamure et al (1997), Moriya et al (1997), Koike et al (1995) the non structural proteins of HCV were cloned directly downstream of the albumin and the MUP promoters used in these system. We preferred to use the HCV

5′ UTR because we thought that transgenic animals could be also used to clarify the role of the 5' UTR belonging to different genotype. In fact, previous in vitro studies (Buratti et al., 1997) have shown that the 5' UTR belonging to different genotypes can have different translation initiation capability. Several studies have demonstrated that HCV genotype of the infecting virus are responsible of the severity of the infection and influence also the response to INF-A.(Pontisso et al., 1995). Accordingly,the type of strategy used to design our transgene construct allowed the easy replacement of the 5' UTR of a viral genotype with that of another one allowing to produce different types of transgenic animal in which the effects of the different 5' UTR could be analysed. However,its presence in our construct used to produce the transgenic animals could play a key role in this hinibition. The activity of a promoter is in fact regulated by several factors and mechanisms that must play in concert to activate its function. The 300 bp of the 5′ UTR localised downstream the transferrin promoter could therefore have interfered in the fine organisation of the promoter for starting the transcription of the transgene. Also in this case, the positive results obtained in vitro show another time the higher complexity of a animal model in comparison with a cellular system.

Nonetheless, the preliminary results obtained in this project show the feasibility of obtaining a system in which a moderate expression of the HCV viral protein could be precisely regulated and it also demonstrated the possible switching on of the expression of a gene of interest only in a limited number of liver cells, which remained rearranged for a long time. These two achievements are absolute requisites for the optimisation of this original HCV transgenic animal system.

REFERENCES

Abremski, K., and Hoess, R. (1984). Bacteriophage P1 site-specific recombination. Purification and properties of the Cre recombinase protein. J Biol Chem *259*, 1509-14.

Abremski, K., Hoess, R., and Sternberg, N. (1983). Studies on the properties of P1 site-specific recombination: evidence for topologically unlinked products following recombination. Cell *32*, 1301-11.

Adrian, G. S., Fischbach, K., Lu, Y., Gayet, O., Rivera, E., and Bowman, B. H. (1990). Expression of chimeric human transferrin genes in transfected human tumor cell lines. SAAS Bull Biochem Biotechnol *3*, 97-101.

Akagi, K., Sandig, V., Vooijs, M., Van der Valk, M., Giovannini, M., Strauss, M., and Berns, A. (1997). Cre-mediated somatic site-specific recombination in mice. Nucleic Acids Res *25*, 1766-73.

Alter, H. J., Purcell, R. H., Shih, J. W., Melpolder, J. C., Hougton, M., Q.L., C., and Kuo, G. (1989). Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. N. Engl. J. Med. *321*, 1494-1500.

Alter, M. J., Morgolis, H. S., Krawczynski, K., Judson, F. N., Maree, A., Hu, P. J., Miller, J. K., Gerber, M. A., Sampliner, E., Meeks, E. L., and Beach, M. J. (1992). The natural history of comunity acquired hepatitis C in the United States. N. Engl. J. Med *327*, 1899-1905.

Anton, M., and Graham, F. L. (1995). Site-specific recombination mediated by an adenovirus vector expressing the Cre recombinase protein: a molecular switch for control of gene expression. J Virol *69*, 4600-6.

Araki, K., Araki, M., Miyazaki, J., and Vassalli, P. (1995). Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. Proc Natl Acad Sci U S A *92*, 160-4.

Barba, G., Harper, F., Harada, T., Kohara, M., Goulinet, S., Matsuura, Y., Eder, G., Schaff, Z., Chapman, M. J., Miyamura, T., and Brechot, C. (1997). Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. Proc Natl Acad Sci U S A *94*, 1200-5.

Bartenschlager, R., Ahlborn-Laake, L., Mous, J., and Jacobsen, H. (1994). Kinetic and stuctural analysis of hepatitis C virus polyprotein processing. J Virol *69*, 5045-5055.

Bartenschlager, R., Ahlborn-Laake, L., Mous, J., and Jacobsen, H. (1993). Nonstructural protein 3 of the hepatitis C virus encodes a serine-type proteinase required for cleavage at the NS3/4 and NS4/5 junctions. J. Virol *67*, 3835-3844.

Berkner, K. L. (1988). Development of adenovirus vectors for the expression of heterologous genes. Biotechniques *6*, 616-29.

Berkner, K. L. (1992). Expression of heterologous sequences in adenoviral vectors. Curr Top Microbiol Immunol *158*, 39-66.

Bett, A. J., Haddara, W., Prevec, L., and Graham, F. L. (1994). An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. Proc Natl Acad Sci U S A *91*, 8802-6.

Bett, A. J., Prevec, L., and Graham, F. L. (1993). Packaging capacity and stability of human adenovirus type 5 vectors. J Virol *67*, 5911-21.

Botarelli, P., Brunetto, M. R., Minutello, M. A., Calvo, P., Unutmaz, D., Weiner, A. J., Choo, Q. L., Shuster, J. R., Kuo, G., Bonino, F., and et al. (1993). T-lymphocyte response to hepatitis C virus in different clinical courses of infection. Gastroenterology *104*, 580-7.

Bout, A., Imler, J. L., Schultz, H., Perricaudet, M., Zurcher, C., Herbrink, P., Valerio, D., and Pavirani, A. (1994). In vivo adenovirus-mediated transfer of human CFTR cDNA to rhesus monkey airway epithelium: efficacy, toxicity and safety. Gene Ther *1*, 385-94.

Bowman, B. H., Jansen, L., Yang, F., Adrian, G. S., Zhao, M., Atherton, S. S., Buchanan, J. M., Greene, R., Walter, C., Herbert, D. C., and et al. (1995). Discovery of a brain promoter from the human transferrin gene and its utilization for development of transgenic mice that express human apolipoprotein E alleles. Proc Natl Acad Sci U S A *92*, 12115-9.

Bradley, D. W., Majnard, J., Popper, H., Cook, E. H., Ebert, J. W., McCaustland, K. A., Schable, C. A., and Fields, H. A. (1983). Posttransfusional non-A, non-B hpatitis;physicochemical properties of two distinct agents. J.Infect.Dis. *148*, 254-265.

Bradley, D. W., McCoustland, K. A., Cook, E. H., Schable, C. A., Ebert, J. W., and Maynard, J. E. (1985). Post-transfusional Non-A non-B hepatitis in chimpanzees. Physicochemical evidence that a tubule forming agent is a small-envelope virus. gastroenterology *85*, 773-779.

Bronson, S. K., Plaehn, E. G., Kluckman, K. D., Hagaman, J. R., Maeda, N., and Smithies, O. (1996). Single-copy transgenic mice with chosen-site integration [see comments]. Proc Natl Acad Sci U S A *93*, 9067-72.

Brown, E. A., Zhang, H., Ping, L. H., and Lemon, S. M. (1992). Secondary sructure of the 5′ UTR of the hepatitis C virus and pestivirus genomic RNAs. NAR *20*, 5041-5045.

Bukh, J. P., R. H.Miller, R. H. (1994). Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proc Natl Acad Sci U S A *91*, 8239-43.

Buratti, E., Gerotto, M., Pontisso, P., Alberti, A., Tisminetzky, S. G., and Baralle, F. E. (1997). In vivo translational efficiency of different hepatitis C virus 5′-UTRs. FEBS Lett *411*, 275-80.

Cereghini, S., Blumenfeld, M., and Yaniv, M. (1988). A liver-specific factor essential for albumin transcription differs between differentiated and dedifferentiated rat hepatoma cells. Genes Dev 2, 957-74.

Chen, P. J., Wang, J. T., Hwang, L. H., Yang, Y. H., Hsieh, C. L., Kao, J. H., Sheu, J. C., Lai, M. Y., Wang, T. H., and Chen, D. S. (1992). Transient immunoglobulin M antibody response to hepatitis C virus capsid antigen in posttransfusion hepatitis C: putative serological marker for acute viral infection. Proc Natl Acad Sci U S A *89*, 5971-5.

Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A non-B viral hepatitis genome. Science *244*, 359-362.

Choo, Q. L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, A., Barr, P. J., Weiner, A. J., Bradley , D. W., Kuo, G., and Hougton (1991). Genetic organisation and diversity of the hepatitis C virus. PNAS *88*, 2451-2455.

Cochet, M., Gannon, F., Hen, R., Maroteaux, L., Perrin, F., and Chambon, P. (1979). Organization and sequence studies of the 17-piece chicken conalbumin gene. Nature *282*, 567-74.

Collett, M. S., Anderson, D. K., and Retzel, E. (1988). Comparisons of the pestivirus bovine viral diarrhoea virus with members of the flaviviridae. J Gen Virol *69*, 2637-43.

Colombo, M., Kuo, G., Choo, Q. L., Donato, M. F., Del Ninno, E., Tommasini, M. A., and Dioguardi, N. (1989). Prevalence of abibodies to hepatitis C virus in Italian patients with hepatocellular carcinoma. Lancet 2, 1006-1008.

D'Souza, E. D. A., Grace, K., Sangar, D. V., Rowlands, D. J., and Clarke, B. E. (1995). In vitro cleavage of hepatitis C virus polyprotéin substrates by purified recombinant NS3 protease. J. Gen. Virol *76*, 1729-1736.

D'Souza, E. D. A., O'Sullivan, Amphlett, E. M., Rowlands, D. J., Sangar, D. V., and Clarke, B. E. (1994). Analysiys on NS3-mediated processing of the hepatitis C virus non-structural region in Vitro. J. Gen.Virol *75*, 3469-3476.

Dedieu, J. F., Vigne, E., Torrent, C., Jullien, C., Mahfouz, I., Caillaud, J. M., Aubailly, N., Orsini, C., Guillaume, J. M., Opolon, P., Delaere, P., Perricaudet, M., and Yeh, P. (1997). Long-term gene delivery into the livers of immunocompetent mice with E1/E4-defective adenoviruses. J Virol *71*, 4626-37.

Dingwall, C., and Laskey, R. A. (1986). Protein import into the cell nucleus. Annu Rev Cell Biol *2*, 367-90.

Dobuisson, J., Hsu, H. H., Cheung, R. C., Greenberg, H. B., Russel, D. G., and Rice, C. M. (1994). Formation of intracellulare localization of hepatitis C virus envelope

glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. J.Virol *68*, 6147-6160.

Doran, S. E., Ren, X. D., Betz, A. L., Pagel, M. A., Neuwelt, E. A., Roessler, B. J., and Davidson, B. L. (1995). Gene expression from recombinant viral vectors in the central nervous system after blood-brain barrier disruption. Neurosurgery *36*, 965-70.

Dusheiko, G. M., Hobbs, K. E., Dick, R., and Burroughs, A. K. (1992). Treatment of small hepatocellular carcinomas. Lancet *340*, 285-288.

Eckart, M. R., Selby, M., Masiarrz, F., Lee, C., Berger, K., Crawford, K., Kuo, G., Houghton, M., and Choo, Q. L. (1993). The hepatitis C virus encodes a serine protease involved in processing of the putative non-structural proteins from the viral polyprotein precursor. BBRC *192*, 399-406.

Engelhardt, J. F., Yang, Y., Stratford-Perricaudet, L. D., Allen, E. D., Kozarsky, K., Perricaudet, M., Yankaskas, J. R., and Wilson, J. M. (1993). Direct gene transfer of human CFTR into human bronchial epithelia of xenografts with E1-deleted adenoviruses. Nat Genet *4*, 27-34.

Engelhardt, J. F., Ye, X., Doranz, B., and Wilson, J. M. (1994). Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. Proc Natl Acad Sci U S A *91*, 6196-200.

Failla, C., Tomei, L., and De Francesco, R. (1995). An amino-terminal domain of the hepatitis C virus NS3 protease is essential for interaction with NS4A. J.Virol *69*, 1769-1777.

Failla, C., Tomei, L., and R., D. F. (1994). Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus non structural proteins. J. Virol. *68*, 3753-3760.

Farci, P., Alter, H. J., Wong, D. C., Miller, R. H., Govindarajan, S., Engle, R., Shapiro, M., and Purcell, R. H. (1994). Prevention of hepatitis C virus infection in chimpanzees after antibody-mediated in vitro neutralization. PNAS *91*, 7792-7796.

Fong, T. L., Shindo, M., Feinstone, S. M., Hoofnagle, J. H., and Di Bisceglie, A. M. (1991). Detection of replicative intermediates of hepatitis C viral RNA in the liver and serum of patients with chronic hepatitis C. J. Clin. Invest *88*, 1058-1060.

Freinstone, S. M., Kapikian, A. Z., Purcell, R. H., Alter, H. J., and Holland, P. V. (1975). Transfusion-associated hepatitis not due to viral hepatitis type A or B. N. Engl J Med *292*, 767-770.

Friedrich, G., and Soriano, P. (1991). Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. Genes Dev *5*, 1513-23.

Fukushi, S., Kurihara, C., Ishiyama, N., Hoshino, F. B., Oya, A., and Katayama, K. (1997). The sequence element of the internal ribosome entry site and a 25-kilodalton cellular protein contribute to efficient internal initiation of translation of hepatitis C virus RNA. J Virol *71(2)*, 1662-1666.

Fukushige, S., and Sauer, B. (1992). Genomic targeting with a positive-selection lox integration vector allows highly reproducible gene expression in mammalian cells. Proc Natl Acad Sci U S A *89*, 7905-9.

Garson, J. A., Tuke, P. W., Makris, M., Briggs, M., Machin, S. J., Preston, F. E., and Tedder, R. S. (1990). Demonstration of viraemia patterns in haemophiliacs treated with
hepatitis-C-virus-contaminated factor VIII concentrates. Lancet *336*, 1022-1025.

Graham, F. L., and Prevec, L. (1991). Manipulation of adenovirus vectors. Methods in Molecular Biology:Gene Transfer and expression protocols, 109.

Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol *36*, 59-74.

Grakoui, A., McCourt, D. W., Wychowski, C., Feinstone, S. M., and Rice, C. M. (1993). Characterisation of the hepatitis C virus encoded serine proteinase:determination of proteinase-dependent polyprotein cleavage. J Virol *67*, 2832-2843.

Gu, H., Marth, J. D., Orban, P. C., Mossmann, H., and Rajewsky, K. (1994). Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting [see comments]. Science *265*, 103-6.

Gu, H., Zou, Y. R., and Rajewsky, K. (1993). Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. Cell *73*, 1155-1164.

Han, J. H., Shyamala, V., Richman, K. H., Brauer, M. J., Irvine, B., Urdea, M. S., Tekamp, Olson, P., Kuo, G., Choo, Q. L., and Houghton, M. (1991). Charcterization of the terminal regions of hepatitis C viral RNA: identification of conserved sequences in the 5′ UTR and poly(A) tails at the 3′end. PNAS *88*, 1711-1715.

Harada, S., Watanabe, Y., Takeuchi, K., Suzuki, T., Katayama, T., Takebe, Y., Saito, I., and Miyamura, T. (1991). Expression of processed core protein of hepatitis C virus in mammalian cells. J Virol *65*, 3015-21.

Hearing, P., Samulski, R. J., Wishart, W. L., and Shenk, T. (1987). Identification of a repeated sequence element required for efficient encapsidation of the adenovirus type 5 chromosome. J Virol *61*, 2555-8.

Herz, J., and Gerard, R. D. (1993). Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice. Proc Natl Acad Sci U S A *90*, 2812-6.

Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M., and Shimotohno, K. (1991). Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. PNAS *88*, 5547-5551.

Hijikata, M., Mizushima, H., Akagi, T., Mori, S., Kakiuchi, N., Kato, N., Tanaka, T., Kimura, K., and Shimotohmo, K. (1993a). Two distinct proteinase activities required for the processing of the putative non-structural precursor protein of hepatitis C cirus. J. Virol *67*, 4665-4675.

Hijikata, M., Mizushima, H., Tanji, Y., Komoda, Y., Hirowatari, Y., Akagi, T., Katon, Kimura, K., and Shimotohno, K. (1993b). Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. PNAS *90*, 10773-10777.

Hiramatsu, N., Hayashi, N., Haruna, Y., Kasahara, A., Fusamoto, H., Mori, C., Fuke, I., Okayama, H., and Kamada, T. (1992). Immunohistochemical detection of hepatitis C virus-infected hepatocytes in chronic liver disease with monoclonal antibodies to core, envelope and NS3 regions of the hepatitis C virus genome. Hepatology *16*, 306-11.

Hoess, R. H., and Abremski, K. (1984). Interaction of the bacteriophage P1 recombinase Cre with the recombining site loxP. Proc Natl Acad Sci U S A *81*, 1026-9.

Hoess, R. H., and Abremski, K. (1985). Mechanism of strand cleavage and exchange in the Cre-lox site-specific recombination system. J Mol Biol *181*, 351-62.

Honda, M., Brown, E. A., and Lemon, S. M. (1996a). Stability of a stem-loop involving the initiatior AUG control the efficiency of internal initiation of translation of hepatitis C virus RNA. RNA *2*, 955-968.

Honda, M., Ping, L. H., Rijinbrand, R. C. A., Amphlett, E., Clake, B. E., Rowlands, D. J., and Lemon, S. M. (1996b). Structural requirements for initiation of translation by internal ribosome entry within genome lnght hepatitis C virus RNA. Virology *222*, 31-42.

Houghton, M., Selby, M., Weiner, A., and Choo, Q. L. (1994). Hepatitis C virus: structure, protein products and processing of the polyprotein precursor. Curr Stud Hematol Blood Transfus, 1-11.

Hsia, P. C., and Seeff, L. B. (1992). Non-A, non-B hepatitis: impact of the emergence of the hepatitis C virus. Adv Intern Med *37*, 197-222.

Huard, J., Lochmuller, H., Acsadi, G., Jani, A., Holland, P., Guerin, C., Massie, B., and Karpati, G. (1995). Differential short-term transduction efficiency of adult versus newborn mouse tissues by adenoviral recombinants. Exp Mol Pathol *62*, 131-43.

Hwang, S. B., Park, K. J., Kim , T. S., Sung, Y. C., and Lai, M. M. C. (1997). Hepatitis C virus NS5B protein is a membrane-associated phospopreotein witha apredominantly perinuclear localisation. Virology *227:*, 439-446.

Idzerda, R. L., Behringer, R. R., Theisen, M., Huggenvik, J. I., McKnight, G. S., and Brinster, R. L. (1989). Expression from the transferrin gene promoter in transgenic mice. Mol Cell Biol *9*, 5154-62.

Jakson, R. J., Howell, M. T., and Kaminski, A. (1990). The novel mechanism of initiation of Picornavirus RNA translation. Trands in Biochemocal Sciences *15*, 477-483.

Kaito, M., Watanabe, S., Tsukiyama-kohara, K., Yamaguchi, K., Kobayashi, Y., Konishi, M., Yokoi, M., Ishida, S., Suzuki, S., and Kohara, M. (1994). Hepatitis C virus particle detected by immunoelectron microscopi study. J. Gen. Viro *75*, 1755-1760.

Kaneko, T., Tanji, Y., Satoh, S., Hijikata, M., Asabe, S., Kimura, K., and Shimotohno, K. (1994). Production of hte two phospoproteins from the NS5A region of the hepatitis C viral genome. BBRC *205*, 320-326.

Kawamura, T., Furusaka, A., Koziel, M. J., Chung, R. T., Wang, T. C., Schmidt, E. V., and Liang, T. J. (1997). Transgenic expression of hepatitis C virus structural proteins in the mouse. Hepatology *25*, 1014-21.

Kelly, T. J., Jr., and Lewis, A. M., Jr. (1973). Use of nondefective adenovirus-simian virus 40 hybrids for mapping the simian virus 40 genome. J Virol *12*, 643-52.

Kim , J. L., Mongerstern, K. A., Lin, C., Fox, T., Dwyer, M. D., Landro, J. A., Chambers, S. P., Markland, W., Lepre, C. A., O'malley, E. T., Herbeson, S. L., Rice, C. M., Murcko, M. A., Corin, P. R., and Thomson, J. A. (1996). Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. Cell *87*, 343-355.

Koike, K., Moriya, K., Ishibashi, K., Matsuura, Y., Suzuki, T., Saito, I., Iino, S., Kurokawa, K., and Miyamura, T. (1995). Expression of hepatitis C virus envelope proteins in transgenic mice. J Gen Virol *76*, 3031-8.

Koike, K., Moriya, K., Ishibashi, K., Yotsuyanagi, H., Shintani, Y., Fujie, H., Kurokawa, K., Matsuura, Y., and Miyamura, T. (1997). Sialadenitis histologically resembling Sjogren syndrome in mice transgenic for hepatitis C virus envelope genes. Proc Natl Acad Sci U S A *94*, 233-6.

Kotwal, G. J., Baroudy, B. M., Kuramoto, I. K., McDonald, F. F., Schiff, G. M., Holland, P. V., and Zeldis, J. B. (1992). Detection of acute hepatitis C virus infection by ELISA using a synthetic peptide comprising a structural epitope. Proc Natl Acad Sci U S A *89*, 4486-9.

Kuhn, R., Schwenk, F., Aguet, M., and Rajewsky, K. (1995). Inducible gene targeting in mice. Science *269*, 1427-9.

Laird, P. W., Zijderveld, A., Linders, K., Rudnicki, M. A., Jaenisch, R., and Berns, A. (1991). Simplified mammalian DNA isolation procedure. Nucleic Acids Res *19*, 4293.

Lakso, M., Steeg, P. S., and Westphal, H. (1992). Embryonic expression of nm23 during mouse organogenesis. Cell Growth Differ *3*, 873-9.

Landfor, R. E., Norvall, L., Chavez, D., White, R., Frenzel, G., Simonsen, C., and Kim, J. (1993). Analysis of hepatitis C virus capsid, E1 and E2/NS1 proteins expressed in insect cells. Virology *197*, 225-235.

Le Gal La Salle, G., Robert, J. J., Berrard, S., Ridoux, V., Stratford-Perricaudet, L. D., Perricaudet, M., and Mallet, J. (1993). An adenovirus vector for gene transfer into neurons and glia in the brain. Science *259*, 988-90.

Lemon, S. M., and Brown, E. A. (1994). Hepatitis C virus and chronic liver disease. Curr Clin Top Infect Dis *14*, 120-41.

Lo, S. Y., Masiarz, F., Hwang, S. B., Lai, M. M., and Ou, J. H. (1995). Differential subcellular localization of hepatitis C virus core gene products. Virology *213*, 455-61.

Lo, S. Y., Selby, M., Tong, M., and Ou, J. H. (1994). Comparative studies of the core gene products of two different hepatitis C virus isolates: two alternative forms determined by a single amino acid substitution. Virology *199*, 124-31.

Lu, H. H., and Wimmer, E. (1996). Poliovirus chimeras replicating under the translationcontrol og genetic elements of Hepatitis C virus reveal unusual properties of the internal ribosome entry site of hepatitisC virus. PNAS USA *93*, 1412-1417.

Lucero, M. A., Schaeffer, E., Cohen, G. N., and Zakin, M. M. (1986). The 5′ region of the human transferrin gene: structure and potential regulatory sites. Nucleic Acids Res *14*, 8692.

Magrin, S., Pinzello, G., Craxi, A., Almasio, P., and Pagliaro, L. (1992). Anti-HCV, anti-GOR, and autoimmunity [letter; comment]. Lancet *339*, 871.

Matsumoto, M., Hsieh, T. Y., Zhu, N., VanArsdale, T., Hwang, S. B., Jeng, K. S., Gorbalenya, A. E., Lo, S. Y., Ou, J. H., Ware, C. F., and Lai, M. M. (1997). Hepatitis C virus core protein interacts with the cytoplasmic tail of lymphotoxin-beta receptor. J Virol *71*, 1301-9.

Matsumoto, M., Hwang, S. B., Jeng, K. S., Zhu, N., and Lai, M. M. (1996). Homotypic interaction and multimerization of hepatitis C virus core protein. Virology *218*, 43-51.

McGrory, W. J., Bautista, D. S., and Graham, F. L. (1988). A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. Virology *163*, 614-7.

Metzger, S., Leff, T., and Breslow, J. L. (1990). Nuclear factors AF-1 and C/EBP bind to the human ApoB gene promoter and modulate its transcriptional activity in hepatic cells. J Biol Chem *265*, 9978-83.

Michel, G., Ritter, A., Gerken, G., Meyer zum Buschenfelde, K. H., Decker, R., and Manns, M. P. (1992). Anti-GOR and hepatitis C virus in autoimmune liver diseases [see comments]. Lancet *339*, 267-9.

Mishiro, S., Hoshi, Y., Takeda, K., Yoshikawa, A., Gotanda, T., Takahashi, K., Akahane, Y., Yoshizawa, H., Okamoto, H., Tsuda, F., and et al. (1990). Non-A, non-B hepatitis specific antibodies directed at host-derived epitope: implication for an autoimmune process [published erratum appears in Lancet 1990 Jan 26;337(8735):252] [see comments]. Lancet *336*, 1400-3.

Mishiro, S., Takeda, K., Hoshi, Y., Yoshikawa, A., Gotanda, T., and Itoh, Y. (1991). An autoantibody cross-reactive to hepatitis C virus core and a host nuclear antigen. Autoimmunity *10*, 269-73.

Morgan (1983). Synthesis and secretion of transferrin promoter. Accademic press, new York *3*, 331-355.

Moriya, K., Yotsuyanagi, H., Shintani, Y., Fujie, H., Ishibashi, K., Matsuura, Y., Miyamura, T., and Koike, K. (1997). Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J Gen Virol *78*, 1527-31.

Myamoto, H., Okamoto, H., Sato, K., Tanaka, T., and Mishiro, S. (1992). Exstraordinarily low density of hepatitis C virus estimated by sucrose density gradient centrifugation and the polimerase chain reaction. J. Gen. Virol *73*, 715-718.

Nasoff, M. S., Zebedee, S. L., Inchauspe, G., and Prince, A. M. (1991). Identification of an immunodominant epitope within the capsid protein of hepatitis C virus. Proc Natl Acad Sci U S A *88*, 5462-6.

Okamoto, H., Munekata, E., Tsuda, F., Takahashi, K., Yotsumoto, S., Tanaka, T., Tachibana, K., Akahane, Y., Sugai, Y., Miyakawa, Y., and et al. (1990). Enzyme-linked immunosorbent assay for antibodies against the capsid protein of hepatitis C virus with a synthetic oligopeptide. Jpn J Exp Med *60*, 223-33.

Okamoto, H., Okada, S., Sugiyama , Y., Kurai, K., Iizuka, H., Machida, A., Miyakawa, Y., and Mayumi, M. (1991). Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. J Gen Virol *72(Pt 11)*, 2697-2704.

Okamoto, H., Tsuda, F., Machida, A., Munekata, E., Akahane, Y., Sugai, Y., Mashiko, K., Mitsui, T., Tanaka, T., Miyakawa, Y., and et al. (1992). Antibodies against synthetic oligopeptides deduced from the putative core gene for the diagnosis of hepatitis virus infection [see comments]. Hepatology *15*, 180-6.

Oldstone, M. B. (1987). Molecular mimicry and autoimmune disease [published erratum appears in Cell 1987 Dec 4;51(5):878]. Cell *50*, 819-20.

Omata, M., and Kato, N. (1994). Recent advances in hepatitis C virus research. J Gastroenterol *29*, 377-82.

Orban, P. C., Chui, D., and Marth, J. D. (1992). Tissue- and site-specific DNA recombination in transgenic mice. Proc Natl Acad Sci U S A *89*, 6861-5.

Pasquinelli, C., Shoenberger, J. M., Chung, J., Chang, K. M., Guidotti, L. G., Selby, M., Berger, K., Lesniewski, R., Houghton, M., and Chisari, F. V. (1997). Hepatitis C virus core and E2 protein expression in transgenic mice. Hepatology *25*, 719-27.

Payne, S. L., Salinovich, O., Nauman, S. M., Issel, C. I., and Montelaro, R. C. (1987). Course and extent of variation of equine infectious anemia virus during parallel persistent infections. J. Vorol *61*, 1266-1270.

Pistello, M., Maggi, F., Vatteroni, L., Cecconi, N., Panicucci, F., Bresci, G. P., Gambardella, L., Taddei, M., Bionda, A., and Tuoni, M. (1994). Prevalence of hepatitis C virus genotypes in Italy. J Clin Microbiol *32*, 232-234.

Pontisso, P., Gerotto, M., Chemello, L., Casarin, C., Tisminetzky, S., Baralle, F., and Alberti, A. (1995). Hepatitis C virus genotypes HCV-1a and HCV-1b: the clinical point of view [letter]. J Infect Dis *171*, 760.

Prevec, L., Campbell, J. B., Christie, B. S., Belbeck, L., and Graham, F. L. (1990). A recombinant human adenovirus vaccine against rabies. J Infect Dis *161*, 27-30.

Ragot, T., Vincent, N., Chafey, P., Vigne, E., Gilgenkrantz, H., Couton, D., Cartaud, J., Briand, P., Kaplan, J. C., Perricaudet, M., and et al. (1993). Efficient adenovirus-mediated transfer of a human minidystrophin gene to skeletal muscle of mdx mice. Nature *361*, 647-50.

Ray, R. B., Lagging, L. M., Meyer, K., and Ray, R. (1996). Hepatitis C virus core protein cooperates with ras and transforms primary rat embryo fibroblasts to tumorigenic phenotype. J Virol *70*, 4438-43.

Reynolds, J. E., Kaminski, A., Kettingnen, H. J., Grace, K., Clarke, B. E., Rowlands, D. J., and Jakson, R. J. (1995). Analysis of locus heterogeneity in Waardenburg syndrome types 1 and 2 using highly informative microsatellite markers. Hum Hered *45(5)*, 243-252.

Rijnbrand, R., Bredenbeek, P., Van der Straten, T., Whetter, L., Inchuspe, G., Lemon, S., and Spaan, W. (1995). Almost the entire 5'UTR of the hepatitis C virus is required for cap-indipendent translation. FEBSlett *365*, 115-119.

Rijnbrand, R. C., Abbink, T. E., Haasnoot, P. C., Spaan, W. J., and Bredenbeek, P. J. (1996). The influence of AUG codons in the hepatitis C virus 5' nontranslated region on translation and mapping of the translation initiation window. Virology *226*, 47-56.

Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, T. Y., Koy, S., Onji, M., Ohta, Y., Choo , Q. L., Houghton, M., and Kuo, G. (1990). Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. PNAS *87*, 6547-6549.

Sakai, K., Mitani, K., and Miyazaki, J. (1995). Efficient regulation of gene expression by adenovirus vector-mediated delivery of the CRE recombinase. Biochem Biophys Res Commun *217*, 393-401.

Sallberg, M., Ruden, U., Wahren, B., and Magnius, L. O. (1992). Immune response to a single peptide containing an immunodominant region of hepatitis C virus core protein: the isotypes and the recognition site. Immunol Lett *33*, 27-33.

Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J., and Roe, B. A. (1980). Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J Mol Biol *143*, 161-78.

Sansonno, D., and Dammacco, F. (1993). Hepatitis C virus c100 antigen in liver tissue from patients with acute and chronic infection. Hepatology *18*, 240-5.

Santolini, E., Migliaccio, G., and La Monica, N. (1994). Biosynthesis and biochemical properties of the hepatitis C virus core protein. J Virol *68*, 3631-41.

Sauer, B., and Henderson, N. (1989). Cre-stimulated recombination at loxP-containing DNA sequences placed into the mammalian genome. Nucleic Acids Res *17(1)*, 147-161.

Sauer, B., and Henderson, N. (1988). Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proc Natl Acad Sci U S A *85(14)*, 5166-517.

Schaeffer, E., Boissier, F., Py, M. C., Cohen, G. N., and Zakin, M. M. (1989). Cell type-specific expression of the human transferrin gene. Role of promoter, negative, and enhancer elements. J Biol Chem *264*, 7153-60.

Selby, M. J., Choo, Q. L., Berger, K., Kuo, G., Glazer, E., Eckart, M., Lee, C., Chien, D., Kuo, C., and Houghton, M. (1993). Expression, identification and subcellular localization of the proteins encoded by the hepatitis C viral genome. J Gen Virol *74*, 1103-13.

Shih, C. M., Lo, S. J., Miyamura, T., Chen, S. Y., and Lee, Y. H. (1993). Suppression of hepatitis B virus expression and replication by hepatitis C virus core protein in HuH-7 cells. J Virol *67*, 5823-32.

Shimizu, Y. K., Hijikata, M., Iwamoto, A., Alter, H. J., Purcell, R. H., and Yoshikura, H. (1994). Neutralizing antibodies against hepatitis C virus and the emergence of neutralization escape mutant viruses. J Virol *68*, 1494-500.

Shimizu, Y. K., Weiner, A. J., Rosenblatt, J., Wong, D. C., Shapiro, M., Popkin, T., Houghton, M., Alter, H. J., and Purcell, R. H. (1990). Early events in hepatitis C virus infection of chimpanzees. PNAS *87*, 6441-6444.

Silini, E., Bono, F., Cividini, A., Cerino, A., Bruno, S., Rossi, S., Belloni, G., Brugnetti , B., Civardi, E., and Salvaneschi, L. (1995). Differential distribution of hepatitis C virus genotypes in patients with and without liver function abnormalities. Hepatology *21*, 285-290.

Simmonds, P., McOmish, F., Yap, P. L., Chan, S. W., Lin, C. K., Dusheiko, G., Saeed, A. A., and Holmes, E. C. (1993). Sequence variability in the 5′ non-coding region of hepatitis C virus: identification of a new virus type and restrictions on sequence diversity. J Gen Virol *74*, 661-668.

Simmons, J. N., Leary, T. P., and Dawson, G. J. (1995). Isolation af novel virus-like sequences associated with human hepatitis. Nature med *1*, 564-569.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol *98*, 503-17.

Spaete, R. R., Alexander, D., Rugroden, M. E., Choo, Q. L., Berger, K., Crawford, K., Kuo, C., Leng, S., Lee, C., Ralston, R., and et al. (1992). Characterization of the hepatitis C virus E2/NS1 gene product expressed in mammalian cells. Virology *188*, 819-30.

Sternberg, N., and Hamilton (1981). Bacteriophage P1 site-specific recombination. Recombination between loxP sites. J Mol Biol *150*, 467-486.

Stratford-Perricaudet, L. D., Briand, P., and Perricaudet, M. (1992). Feasibility of adenovirus-mediated gene transfer in vivo. Bone Marrow Transplant *1*, 151-2.

Suzuki, R., Matsuura, Y., Suzuki, T., Ando, A., Chiba, J., Harada, S., Saito, I., and Miyamura, T. (1995). Nuclear localization of the truncated hepatitis C virus core protein with its hydrophobic C terminus deleted. J Gen Virol *76*, 53-61.

Swift, G. H., Kruse, F., MacDonald, R. J., and Hammer, R. E. (1989). Differential requirements for cell-specific elastase I enhancer domains in transfected cells and transgenic mice. Genes Dev *3*, 687-96.

Takehara, T., Hayashi, N., Miyamoto, Y., Yamamoto, M., Mita, E., Fusamoto, H., and Kamada, T. (1995). Expression of the hepatitis C virus genome in rat liver after cationic liposome-mediated in vivo gene transfer. Hepatology *21*, 746-51.

Tanaka, T., Kato, N., Cho, M. J., Sugiyama, K., and Shimotohno, K. (1996). Structure of the 3′ terminus of the hepatitis C virus genome. J of Virol *70*, 3307-3312.

Tapscott, S. J., Lassar, A. B., and Weintraub, H. (1992). A novel myoblast enhancer element mediates MyoD transcription. Mol Cell Biol *12*, 4994-5003.

Theisen, M., Behringer, R. R., Cadd, G. G., Brinster, R. L., and McKnight, G. S. (1993). A C/EBP-binding site in the transferrin promoter is essential for expression in the liver but not the brain of transgenic mice. Mol Cell Biol *13*, 7666-76.

Thomssen, R., Bonk, S., Propfe, C., Heerman, K. H., Kochel, H. G., and Uy, A. (1992). Association of hepatitis C virus in human sera with B-lipoprotein. Med Microbiol Immunol *181*, 293-300.

Tomei, L., Failla, C., Santolini, E., De Francesco, R., and La Monica, N. (1993). NS3 is a serine protease required for processing of hepatitis C virus polyprotein. J Virol *67*, 4017-26.

Tsukiyama-Kohara, K., Lizuka , N., Kohara, M., and Nomoto, A. (1992). Internal ribosome entry site within hepatitis C virus RNA. Journal of virology *66*, 1476-1484.

Wang , C., Le , S. Y., Ali, N., and Siddiqui, A. (1995). An RNA pseudoknot is an essential structural element of the internal ribosome entry site located within the hepatitis C virus 5' noncoding region. RNA *1(5)*, 526-537.

Wang, C., Sarnow, P., and Siddiqui, A. (1994). A conserved helical element is essential for internal initiation of translation of hepatitis C virus RNA. J Virol *68*, 7301-7.

Wang, C., Sarnow, P., and Siddiqui, A. (1993). Translation of human hepatitis C virus RNA in cultured cells is mediated by an internal ribosome-binding mechanism. J.of Virol *67*, 3338-3344.

Wang, J., Ma, Y., and Knechtle, S. J. (1996). Adenovirus-mediated gene transfer into rat cardiac allografts. Comparison of direct injection and perfusion. Transplantation *61*, 1726-9.

Wang, Y., Krushel, L. A., and Edelman, G. M. (1996). Targeted DNA recombination in vivo using an adenovirus carrying the cre recombinase gene. Proc Natl Acad Sci U S A *93*, 3932-6.

Xu, L. Z., Lardul, D., Delaporte, E., Brechot, C., and Kremsdorf, D. (1994). Hepatitis C virus genotype 4 is highly prevalent in central Africa (gabon). J.Gen.Virol *75:*, 2393-2398.

Yamada, M., Kakumu, S., Yoshioka, K., Higashi , Y., Tanaka, K., Ishikawa, T., and Takayanagi, M. (1994). Hepatitis C virus genotypes are not responsible for development of serious liver disease. Dig Dis Sci *39*, 234-239.

Yang, Y., Ertl, H. C., and Wilson, J. M. (1994). MHC class I-restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. Immunity *1*, 433-42.

Yang, Y., Jooss, K. U., Su, Q., Ertl, H. C., and Wilson, J. M. (1996). Immune responses to viral antigens versus transgene product in the elimination of recombinant adenovirus-infected hepatocytes in vivo. Gene Ther *3*, 137-44.

Yang, Y., Li, Q., Ertl, H. C., and Wilson, J. M. (1995). Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. J Virol *69*, 2004-15.

Yang, Y., Nunes, F. A., Berencsi, K., Furth, E. E., Gonczol, E., and Wilson, J. M. (1994). Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. Proc Natl Acad Sci U S A *91*, 4407-11.

Yang, Y., Su, Q., and Wilson, J. M. (1996). Role of viral antigens in destructive cellular immune responses to adenovirus vector-transduced cells in mouse lungs. J Virol *70*, 7209-12.

Yang, Y., Xiang, Z., Ertl, H. C., and Wilson, J. M. (1995). Upregulation of class I major histocompatibility complex antigens by interferon gamma is necessary for T-cell-mediated elimination of recombinant adenovirus-infected hepatocytes in vivo. Proc Natl Acad Sci U S A *92*, 7257-61.

Yen, J. H., Chang, S. C., Hu, C. R., Chu , S. C., Lin , S. S., Hsieh, Y. S., and Chang, M. F. (1995). Cellular proteins specifically bind to the 5'-noncoding region of hepatitis C virus RNA. Virology *208(2*, 723-732.

Yuasa, T., Ishikawa, G., Manabe, S. I., Sekiguchi, S., Takeuchi, K., and Miyamura, T. (1991). The particle size of hepatitis C virus estimated by filtration through microporous regenerated cellulose fibre. J. Gen. Virol *72*, 2021-2024.

Zhang, Y., Riesterer, C., Ayrall, A. M., Sablitzky, F., Littlewood, T., and Reth, M. (1996). inducible site-directed recombination in mouse embryonic stem cells. Nucleic Acids research, 543-548.

Zou, Y. R., Gu, H., and Rajewsky, K. (1993). Generation of a mouse strain that produces immunoglobulin kappa chains with human constant regions. Science *262*, 1271-1274.