Mapping of the hepatitis B virus genome in hepatocellular carcinoma using PCR and demonstration of a potential trans-activator encoded by the frequently detected fragment

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The association of hepatitis B virus (HBV) infection with hepatocellular carcinoma (HCC) is well established. Insertional mutagenesis, trans-activation by truncated X or preS2/S regions and activation of growth regulatory genes or oncogenes have all been suggested as possible mechanisms for this carcinogenesis. However, no consensus regarding the mechanism or region of the HBV genome involved has been established. Of the 36 HCC tissues analysed for the presence and extent of the HBV genome, using multiple overlapping PCR, 22 (61%) were found to be positive. Twenty of these showed the presence of a fragment (nucleotides 636 to 746) that covered part of the surface antigen gene. The recognized trans-activators, X and preS2/S, were present in only seven (31.8%) and 12 (54.5%) cases, respectively. In two cases the entire viral genome was detected. The trans-activation potential of the cloned S fragment (nucleotides 426 to 851) covering the frequently detected fragment (nucleotides 636 to 746) was investigated in co-transfection experiments. This fragment was able to trans-activate the HBV enhancer–X promoter target. To define the specificity of the trans-activation and the sequences involved, frameshift and deletion mutants of this fragment were constructed and analysed. The trans-activation activity was lost in the frameshift mutants. The deletion mutants that retained nucleotide sequences 436 to 679 showed trans-activation activity whereas the other ones (nucleotide sequences 436 to 611) did not show any activity. It is suggested that the frequently detected HBV genome fragment belonging to the S gene frame has a trans-activation potential. This may explain the mechanism for pathogenicity of HBV-associated HCC.

Introduction

Hepatocellular carcinoma (HCC) is a common malignancy occurring in many parts of the world and is estimated to be responsible for more than 250,000 deaths annually (Zhou et al., 1989). There is strong epidemiological evidence of a causal relationship between persistent hepatitis B virus (HBV) infection and development of HCC. HBV carriers are at a 200-fold greater risk of developing HCC as compared with non-carriers (Beasley, 1982). Molecular hybridization studies have demonstrated the presence of HBV DNA integrated in the host chromosomes in a large proportion of HCC tissue and this DNA may act as an insertional mutagen (Moroy et al., 1986). The integration occurs at random and brings about extensive alterations in the viral genome such as micro-deletions and translocations (Nakamura et al., 1988). Micro-deletions have been suggested to be the most common change leading to altered host gene functions (Rogler et al., 1985). Such aberrant host gene expression has been demonstrated with reference to epidermal growth factor (Heldin & Westermark, 1984), insulin-like growth factor (Fu et al., 1988), a retinoic acid receptor (de The et al., 1987), cyclin A (Wang et al., 1990) and oncogenes such as C-myc and N-ras (Fourel et al., 1990; Himeno et al., 1988; Dejean et al., 1986). Recent studies have shown that two gene products of HBV, namely the X and truncated preS2/S polypeptides, can trans-activate viral and cellular promoters. The relatively higher frequency of integration of preS2/S than X suggests that the trans-activating property of integrated preS2/S sequences is the most plausible mechanism for HBV-associated carcinogenesis (Takada et al., 1990; Kekule et al., 1990). Most such trans-activation studies involved single HBV integrated inserts. Therefore, it is difficult to address the importance of such mechanisms in the aetiology of all HBV-related HCCs. The present study investigates 36 HCC tissue samples in order to determine the presence and extent of the HBV genome in each using multiple overlapping PCR spanning almost
the entire length of the HBV genome. It further demonstrates the trans-activating potential of the HBV genome S fragment (nucleotides 426 to 851) which is detected frequently in HCC tissue. In addition, it investigates the effect of frameshift mutations and 3' end deletions in order to analyse the exact frame and location of the trans-activator.

Methods

**Human liver samples.** Human hepatoma tissues were obtained either by needle biopsy (32 cases) or during therapeutic resection (four cases) after obtaining informed consent. Each of the samples was divided into two parts: one part was immediately frozen in liquid nitrogen and stored at -70 °C until it was used for DNA isolation and analysis and the other was fixed in formalin and processed for routine histopathological examination which confirmed the diagnosis.

**HBV serological markers.** Five ml of blood was collected from each patient, the serum from which was separated under aseptic conditions and stored at -70 °C until further use. Serological markers for HBV infection including hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), antibody to HBsAg (anti-HBs), antibody to HBeAg (anti-HBe) and antibody against hepatitis B core antigen (anti-HBc) were tested according to the manufacturer’s guidelines using commercial microELISA test kits (Organon Teknika).

**Detection of HBV DNA in serum by dot-blot hybridization.** Dot-blot hybridization was performed on the serum for HBV DNA detection as described previously (Panda et al., 1991) using nylon membranes (NY-13N, Nytran, Schleicher & Schuell) and full-length HBV DNA (adw3) probe labelled with [α-32P]dCTP (3000 Ci/mmole; Bhabha Atomic Research Center, Bombay, India) by random primer oligolabelling (Feinberg & Vogelstein, 1985). The specific activity of the probe was 10⁸ to 5 × 10⁹ c.p.m./μg DNA.

**HBV primers for PCR.** Pairs of oligonucleotide primers (Table 1) used to amplify different regions of HBV were either chosen from published sequences (Baginski et al., 1990) or by analysis of the genome using OLIGO software version 4.0 and included sequences conserved among different HBV subtypes. The oligonucleotides were synthesized on an automated DNA synthesizer (Model 391B, Applied Biosystems) using phosphoramidite chemistry. They were deprotected, cleaved and purified by the standard protocol.

**Isolation of DNA from liver samples.** High M₆ DNA was extracted from the liver tissues according to the method of Brechot et al. (1981) with slight modifications. Briefly, tissue specimens were pulverized in liquid nitrogen, transferred to lysis buffer (10 mM-Tris-HCl pH 7.8, 10 mM-EDTA, 10 mM-NaCl, 0.5% SDS) and digested overnight at 37 °C with 100 μg/ml proteinase K (Boehringer Mannheim). The DNA solution was extracted once each with buffer-saturated phenol and chloroform:isoamyl alcohol (24:1) followed by precipitation with ammonium acetate and ethanol. The DNA precipitate was redissolved in TE (10 mM-Tris–HCl and 1 mM-EDTA, pH 8.0) and digested with 100 μg/ml RNase A (Sigma) for 3 h at 37 °C and then the extraction procedure was repeated. The purified DNA was redissolved in 50 μl TE, quantified by A₂₆₀ (using a Philips Pye Unicam 8800) and stored at

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**Table 1. Sequences of oligonucleotide primers used for overlapping PCR and amplification products’ size**

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Subgenomic analysis of HBV in HCC

Fig. 1. (a) PCR detection of the surface antigen region of HBV DNA (nucleotides 426 to 851) in liver tumour tissues. Ethidium bromide staining of the amplification products with primers MD14 and MD13. Lane 1, pBR322/Sau3A marker; lanes 2 to 8 and 10 to 11, samples; lane 9, positive control, recombinant HBV adw<sub>2</sub>; lane 12, negative control. (b) Southern blot analysis and hybridization with a <sup>32</sup>P-labelled probe. Lanes 1 to 7 and 9 to 10, samples; lane 8, positive control, recombinant HBV adw<sub>2</sub>.

PCR amplification and detection of HBV DNA. Amplification of HBV DNA was carried out according to the method described by Larzul et al. (1988). Briefly, amplification was performed in a 50 µl reaction mixture containing 1 µg tumour DNA, 10 mM-Tris-HCl pH 8.3, 1.5 to 4.0 mM-MgCl<sub>2</sub> (depending upon the primer pair used), 0.01% Triton X-100, 50 pmol of each primer, 200 µM of each dNTP (dATP, dCTP, dGTP or dTTP) and 2.5 units Taq polymerase (Promega or USB). After overlaying with 50 µl mineral oil (USB), the amplifications were carried out for 32 to 37 cycles (95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 to 3 min) depending upon the template to be amplified, followed by a 10 min final extension. For nested PCR, 1 to 5 µl of the first PCR product was used as a template and reamplified with internal primers in order to increase the sensitivity and specificity of detection. In all the PCR reactions, cloned HBV (adw<sub>2</sub>) was used as a positive control. DNA samples from normal liver as well as reaction mixtures without any template DNA were used as negative controls. False positive results were avoided by strict application of the published control measures (Kwok & Higuchi, 1989).

Ten µl of the amplified product was subjected to electrophoresis on a 2% agarose gel (ICN Chemicals) followed by staining with ethidium bromide and visualization under u.v. light (Fig. 1a). For Southern blot hybridization (Southern, 1975) the electrophoresed PCR products were transferred onto a nylon membrane (NY-13N, Nytran, Schleicher & Schuell) by vacuum blotting (LKB Pharmacia). The filters were prehybridized in 50% formamide, 5 x Denhardt's solution (1 x : 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 6 x SSC (1 x : 150 mM-sodium chloride, 15 mM-sodium citrate), 0.5% SDS and calf thymus DNA (100 µg/ml) at 42 °C overnight and hybridized in the same prehybridization solution at 42 °C with <sup>32</sup>P-labelled genome-length HBV (adw<sub>2</sub>) DNA. The filters were then washed under stringent conditions in 0.1 x SSC, 0.1% SDS for 60 min at 68 °C and exposed to Kodak X-Omat AR5, with intensifying screens for 16 to 24 h at −70 °C (Fig. 1b). Wherever internal oligonucleotides were available the specificity of the amplified fragment was confirmed using <sup>32</sup>P-end-labelled primers (Sambrook et al., 1989).

Trans-activation analysis: plasmid constructs. All the constructs were made by standard recombinant DNA techniques. The S fragment of the genome, spanning nucleotides 426 to 851, was amplified by PCR and cloned into plasmid vector Bluescript (pBS<sup>+</sup>, Stratagene). This fragment was further subcloned into eukaryotic expression vector pSG5 (Green et al., 1988) which contains the simian virus 40 enhancer and early promoter to yield plasmid pSG41 (Fig. 2). The deletion mutants from the 3' end were prepared by double digestion with BamHI and BstXI (pSG42, map site 611) or BamHI and SpeI (pSG43, map site 679) (Fig. 3). After end-filling with the Klenow polymerase (Amersham International) they were religated. The frameshift mutants, pSG14a (−1 shift) and pSG14b (−2 shift) were created by site-directed mutagenesis (SDM) (Fig. 3), using a PCR-based extension method with
AAT TCA TAT GGC CCG TTT GTC CTC G 3'; and MD pSG5. The sequences of all the constructs were confirmed by the amplified frameshift mutants were cloned at the constructs. The wild-type is shown at the top of the figure. The broad TCA TGT TGG CCC GTT TGT CCT CTA 3'; MD 14a: 5' GCG AAT TCA ATG TGC CCG TTT GTC CTC TA 3'). The modified primers MD 14a and MD 14b. (MD 14: 5' GCG AAT TCA TGT TGG CCC GTT TGT CCT CTA 3'; MD 14a: 5' GCG AAT TCA TAT GGC CCG TTT GTC CTC G 3'; and MD 14b: 5' GCG AAT TCA ATG TGC CCG TTT GTC CTC TA 3'). The amplified frameshift mutants were cloned at the EcoRI – BamHI site of pSG5. The sequences of all the constructs were confirmed by the dideoxynucleotide sequencing method (Sanger et al., 1977) using the Sequenase version 2.0 sequencing kit (USB).

**Cell culture and DNA transfection assays.** Human hepatoma Huh-7 cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, at 37 °C in 7% CO₂-air. Transfection of plasmid DNA was performed by the calcium phosphate coprecipitation method described elsewhere (Gorman et al., 1983) with 10 cm plates of cells at 60 to 70% confluence. Total DNA concentrations of 40 µg per plate were maintained with plasmid PBS. In the transfection assays 1 µg of pXCAT or pHNLuc reporter plasmid and a 10-fold molar excess of activator plasmid was used. Plasmid pHNLuc (Fig. 2) contains the bacterial luciferase gene under the control of the HBV enhancer and X promoter (HpaI–Neol fragment; HBV adw, nucleotides 966 to 1375). Plasmid pXCAT containing the chloramphenicol acetyltransferase (CAT) gene under the control of the HBV enhancer–X promoter was a kind gift from Dr. Vijay Kumar, ICGEB, India. pCH1100 (1 µg; Stratagene) was used as an internal control for transfection efficiency. Cells were harvested 40 to 48 h post-transfection and assayed for luciferase and CAT activities as previously described (de Wet et al., 1987). For each experiment at least two different preparations of cesium chloride gradient-purified plasmid were used. The expression of the nucleotides 436 to 851 fragment for the S frame was checked using ELISA with a commercial HBsAg detection kit (Organon Teknika) as well as with antibodies to a synthetic peptide (amino acids 124 to 147) of HBsAg.

**Fig. 2.** Schematic representation of transfection assay. (a) PCR-amplified S fragment (nucleotides 426 to 851) with EcoRI–BamHI restriction sites. (b) Cloning of the fragment in the eukaryotic expression vector pSG5, to yield pSG41. (c) Cotransfection of Huh-7 cells with pSG41 and reporter plasmid pHNLuc.

**Fig. 3.** Schematic representation of pSG41 and variant HBV S gene constructs. The wild-type is shown at the top of the figure. The broad arrows refer to the boundaries employed in mutational analysis and the extent of deletion. Open triangles denote the position of the mutation-containing insertions (frameshifts in pSG14a and pSG14b).

**Results**

Fifteen of the 30 cases studied for HBV serology showed evidence of HBV infection (Table 2). Eight were positive for HBsAg and seven for anti-HBV antibodies (three for anti-HBs, one for anti-HBc and three for anti-HBe; Table 2). Of the 36 HCCs screened by overlapping PCR, 22 were positive for one or more regions of the HBV genome (Fig. 4). Two of these (cases 17 and 18) contained the entire HBV genome, whereas the remaining 20 cases showed deletions in one or more regions of the genome. Of these, a small region covering bases 636 to 746 of the HBV genome was observed in 20 of 22 cases, making this the most frequently detected region in the HCCs examined. The X region was covered by four amplified fragments, from nucleotides 130 to 851, 426 to 851, 636 to 746 and 636 to 1420, to detect possible deletions and rearrangements. Since the nucleotides 130 to 851 fragment was present in only two cases, the nucleotides 426 to 851 fragment in 12 cases and the nucleotides 636 to 1420 fragment in seven cases, most of the cases involved extensive deletions of the 5' and 3' ends of the S gene. One case showed possible insertion in the S region, as seen by an increase in the size of the amplified fragment, and was found to have a normal genome sequence (Fig. 1a, lane 5). The ‘X’ region was amplified as two overlapping fragments, nucleotides 1389 to 152 which cover the preS1 and preS2 regions and was found to have a normal genome sequence (Fig. 1a, lane 5). The ‘X’ region was amplified as two overlapping fragments, nucleotides 1389 to 152 which cover the preS1 and preS2 regions and
Table 2. HBV serological status

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* Serum DNA was tested by dot hybridization.
† PCR on DNA extracted from tumour tissue.
‡ ND, Not determined.

nucleotides 2823 to 3199 which cover only the preS2 region. Both the fragments were detected in a total of nine cases, whereas in three cases only the preS2 region was present (Fig. 4). One case (no. 9) had only the preS2 region and no other fragment of the genome could be amplified (Fig. 4). One of the 22 cases was positive for both the X and preS2 regions (nucleotides 1389 to 1630 and 2623 to 152) without any serological markers or any other region of the HBV genome. The C region was detected by amplification of nucleotides 1858 to 2377 and was present in only seven cases.

3' end of the S region shows trans-activator activity

Since the S region (nucleotides 636 to 746) was found to be most frequently present in HBV-positive HCCs, its trans-activation potential was analysed using a fragment larger than but covering this region. Initially, in three separate cotransfection experiments (Table 3), using the HBV enhancer and X promoter transcription control elements as targets and luciferase as reporter (Fig. 2) a trans-activation of about threefold (Table 3) was observed. For further confirmation, another reporter system (pXCAT) was used for the transfection assay and a similar result with an average of threefold trans-activation over the background level (Fig. 5) was

Table 3. Trans-activation of HBV enhancer/X protein by the S (amino acids 102 to 226) polypeptide

<table>
<thead>
<tr>
<th>Co-transfecting plasmid*</th>
<th>Luciferase (counts)†</th>
<th>Activation (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µg pSG5</td>
<td>521790</td>
<td>(1)</td>
</tr>
<tr>
<td>5 µg pSG41</td>
<td>1708268</td>
<td>(3.3)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µg pSG5</td>
<td>75434</td>
<td>(1)</td>
</tr>
<tr>
<td>5 µg pSG41</td>
<td>271686</td>
<td>(3.6)</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>85752</td>
<td>(1)</td>
</tr>
<tr>
<td>1 µg pSG41</td>
<td>87475</td>
<td>1.0</td>
</tr>
<tr>
<td>5 µg pSG41</td>
<td>171452</td>
<td>2.0</td>
</tr>
<tr>
<td>10 µg pSG41</td>
<td>27157</td>
<td>0.3</td>
</tr>
<tr>
<td>20 µg pSG41</td>
<td>6906</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* Transfections were carried out in Huh-7 cells with 5 µg pHNLuc and the plasmid indicated.
† For luciferase assay, 20 µg protein was used in experiment 1 and 10 µg protein was used in experiments 2 and 3.
observed. Variability in the absolute luciferase counts between different experiments can be explained by variation in transfection efficiency. However, since the control plasmid pSG5 and the experimental plasmid pSG41 were cotransfected with the reporter plasmid pHNLuc under identical conditions, their comparison is valid within each experiment. At high concentrations of the putative trans-activator, repression of the HBV enhancer and promoter elements was observed (Table 3, experiment 3). This is similar to effects observed with two other well characterized trans-activators, C/EBP and C-jun, on these transcription control elements (Trujillo et al., 1991).

**Trans-activator activity is a property/function of the S open reading frame (ORF)**

To investigate and assign the trans-activator activity as the function of the S ORF and not the P or other ORF, two frameshift mutants (pSG14a and pSG14b) were constructed (Fig. 3) from cloned wild-type HBV DNA (adw). The frameshifts were created at positions −1 and −2 of the start codon present 5’ to the cloned S region. The pSG14a and pSG14b constructs along with pSG41 were tested in cotransfection experiments using pXCAT as a reporter plasmid. No stimulation of CAT gene expression was observed with either pSG14a or pSG14b. However, plasmid pSG41 caused a threefold trans-activation of the CAT reporter gene (Fig. 6). Additional evidence came from the detection of the S antigen peptide in the lysate of cells transfected with pSG41, using both commercial microELISA and an anti-peptide-based ELISA. Therefore these results suggest that the trans-activator activity is a function of the S ORF.

**Trans-activator function is retained after minor deletion of the 3’ terminus of the S fragment**

To identify the region of the S gene responsible for the observed trans-activation, two deletion constructs (pSG42, pSG43) with 3’ ends at positions 611 and 679 were analysed (Fig. 6). The functional consequences of the deletion mutant pSG42 (3’ end truncated at position 611) and mutant pSG43 (3’ end truncated at position 679) were evaluated in cotransfection experiments using pXCAT as a reporter plasmid. Deletion up to nucleotide 679 did not affect trans-activator function whereas an additional deletion of 68 bases up to nucleotide 611 led to loss of its activity (Fig. 6).

**Discussion**

There is a strong epidemiological correlation between HBV and HCC occurrence (Zhou et al., 1989; Szmuness, 1978). The pathogenetic mechanisms involved in the
HBV-associated oncogenic processes remain controversial. Two hypotheses have been put forth. The first is based on the progression of chronic liver damage in the development of carcinoma (Chisari et al., 1989). In the second hypothesis, the virus is proposed to play a more direct role because of its integration leading to insertional mutagenesis (Moroy et al., 1986) or activation of growth control genes by viral trans-activators (Fu et al., 1988). Chronic liver cell damage is usually associated with the expression of viral antigens. In chronic liver disease, expression of HBcAg and the associated cytotoxic T cell response to it are supposed to be the cause of liver cell death (Chen et al., 1988). In contrast HCC cases rarely express viral antigens (Kostich & Ingham, 1977). Several studies on the analysis of subgenomic HBV fragment integration demonstrated the relative paucity of the core region (Chen et al., 1988) whose expression in general is supposed to be essential for chronic liver damage. This suggests that continuing liver cell necrosis, in general, may not explain the development of carcinoma. However, this may be responsible for selection pressure, which leads to evolution of liver cells devoid of the core region but having HBsAg region genomic components.

Several earlier studies have investigated the status (Shafritz & Kew, 1981), subgenomic fragments (Chen et al., 1988; Ziemer et al., 1985) and flanking sequences (Takada et al., 1990) of HBV in HCCs to determine the mode of carcinogenesis. These studies used Southern blot analysis which required more tissue, was less sensitive and did not demonstrate exact deletions or insertions in the HBV genome that occur in a large number of cases. The reports, which were mostly limited to single cases, led to the identification of two trans-activators (Takada & Koike, 1990; Kekule et al., 1990). A study using serologically negative HCCs demonstrated by multiple PCR the presence of various regions of HBV, parts of which were transcriptionally active (Paterlini et al., 1990).

In the present study, by using multiple overlapping fragment amplifications we detected subgenomic regions of HBV in 61% (22 of 36 cases) of the HCCs (Fig. 4), independent of the HBV serological status. Of these cases, only 32% (seven of 22 cases) were positive for the core region, confirming earlier observations (Chen et al., 1988; Takada et al., 1990) of its under-representation in HCCs. Regions encoding previously recognized HBV trans-activators, X and preS2, were detected in 63.6% (14 of 22) of the HBV-positive cases examined. This subset of cases also included all the cases that were positive for the core region. Neither the anti-core cytotoxic T cell response nor the X- or preS2-based trans-activation function alone can, therefore, be universally responsible for the development of HBV-related HCC. In this study, the most commonly detected subgenomic region was part of the S gene, present in 91% (20 of 22 cases) of the HCCs. Part of the S genome region slightly larger than but covering the frequently present fragment, encoding amino acids 102 to 226 of the major S protein, was found to be capable of trans-activating a homologous viral transcription control element. Although this effect was modest, it establishes a potential function for this region. This is the first report in which a relatively large number of HCCs have been examined and a potential function assigned to the most frequently present subgenomic fragment of HBV. Although it is too premature to speculate on the mechanism of this trans-activation, it most likely occurs through protein–protein interactions between the major S polypeptide and some direct transcription factors. Recently, preS2-mediated trans-activation was shown to be due to activation of a transcription factor, NFXB, by the preS2 polypeptide at the endoplasmic reticulum membrane, perhaps through the protein kinase C signalling pathway (Meyer et al., 1992). More direct in vitro studies of the expression and localization of the novel trans-activator identified here may lead to an understanding of its function. Based on the findings of this report, however, we propose that HBV trans-activators play a more significant role in the development of HCC than is currently realized.

Among those examined, seven of the 22 cases positive for HBV DNA were also positive for anti-HBV antibodies (anti-HBs, anti-HBc or anti-HBe) (Table 2). Simultaneously, six of these cases were negative for all viral markers. Similar observations have been made by Paterlini et al. (1990). This raises a fundamental question regarding the risk calculation of hepatoma in hyper-endemic areas for HBV. For countries where a large proportion of the population is positive for anti-HBV antibodies, the prediction of developing carcinoma should take into account the carriers as well as those seroconverted individuals.

We are indebted to Dr A. Siddiqui (University of Colorado School of Medicine, Denver, Co., U.S.A.) for help with trans-activator experiments and to Rajesh Dixit for technical assistance. This work was supported by grants from the Indian Council of Medical Research under the Indo-German Project (No. ICMR 49). S.J. was a recipient of a Biotechnology Career Fellowship from the Rockefeller Foundation and R. R. is a recipient of a CSIR Fellowship, New Delhi, India.

References


(Received 20 August 1993; Accepted 16 September 1993)