MATERIALS AND METHODS
MATERIALS

Acrylamide (Spectrochem), Acetic acid, Acetone, Ammonium persulfate, Agaragar (Qualigens), Agarose, Agarose (low melting point), Ampholines (3.5-9.5; 5-7) (Sigma), Ampicillin, bis Acrylamide (Spectrochem), Bactotryptone, Bactoagar (Difco), β-mercaptoethanol, Bovine serum albumin (Sigma); Boric acid (Qualigens), Bromophenol blue, Bromodeoxyuridine (Sigma), Chloroform (Qualigens), Cassette with intensifying screen (Indu), CSPD (Boehringer Mannheim), Developer (Rege cine films), Dimethyl sulfoxide (Sigma), DNA Digoxigenin labelling and detection kit (Boehringer Mannheim), DH5-α (Courtsey Prof. Das, JNU), Ethylene diamine tetra acetate sodium salt (EDTA) (Qualigens), Ethanol (Bengal chemicals), Ethidium bromide (Sigma), Foetal calf serum (Biological Industries Limited), Formaldehyde (Qualigens), Formamide (Sigma), Fixer (Rege cine films), Glycerol, Glycine (Qualigens), Glutaraldehyde, Hoechst 33258, HEPES (Sigma), Hydroxy-quinoline, Isoamylalcohol, Isopropanol, Methanol, Ethanol (Qualigens), Maleic acid (CDH), Molecular weight protein markers (SDS7 and SDS17) (Sigma), Nitrocellulose filters (0.22 μm) (Millipore), NP-40 (Sigma), Disodium hydrogen phosphate (Qualigens), Nitrocellulose paper (Sigma), Nylon membranes: positively charged (Boehringer Mannheim), Ponceau-S, Penicillin (Sigma), Paraformaldehyde, Crystallised Phenol (Qualigens), Proteinase K (Sigma), Potassium Dihydrogen phosphate (Lobo chemicals), Potassium chloride (Qualigens), RPMI-1640 Medium (Biological industries Ltd., Israel and Gibco-BRL, USA), Restriction enzymes (New England Biolabs, Bangalore Genei), RNase A (Sigma), Sodium dodecyl sulfate (Merck), Sodium carbonate, Sodium bicarbonate, Sodium chloride, Sodium acetate, Sodium hydroxide, tri-Sodium citrate (Qualigens), Sodium lauryl sarcosine, Streptomycin
(Sigma), Tris buffer, TEMED, Trypan blue, Tryptone, Triton-X-100 (Sigma), Tween 20 (CDH), Whatmann 1M & 3M, Xylene Cyanol (Sigma), X-ray films (Indu), Yeast extract (Difco).

Cell Lines

Bloom Syndrome B-lymphoblastoid cell line, GM 03403 E, was procured from Coriell cell repositories, Camden, New Jersey, USA.

Normal B-lymphoblastoid cell line, GA<sub>3</sub>, was procured from National Institute of Immunology, New Delhi, India.

Samples

Serum, as a source of primary antibodies, used in detection of the cancer associated antigen(s) on the cell surface of carcinogen exposed BS-B-lymphoblastoid cells, was separated from the blood samples of cancer patients. The sera samples were obtained from A.I.I.M.S, and Army Hospital, New Delhi. The clinical diagnosis and classification of the various cancers was made by experienced oncologists at these hospitals. The blood samples were collected from cancer patients with -

1. Hodgkin’s Lymphoma
2. Non Hodgkin’s Lymphoma
3. Multiple Myeloma
4. Ovarian cancer
5. Small cell carcinoma
6. Kidney cancer
7. Cervix cancer
DNA Probes

(i) pH60 (0.8 kb) was a human genomic probe cloned in pUC 18 and was obtained from Medical Research Centre (MRC), London. It represented Joining (J), Jγ1, gene segment of T cell receptor gamma gene.

(ii) Tumor suppressor gene, p53, probe, a 1.8kb fragment cloned in pCMV - Neo-Bam vector, was kindly provided to us by Dr. Band, Harvard Medical School.

(iii) The c-myc, 1.8kb fragment (Dalla Favera et al., 1982) representing the 3' region of the human c-myc gene was isolated from the vector pBR 322.

(iv) The 2kb c-myb genomic fragment (human) (Franchini et al., 1983) was purified from F8 plasmid. (All these oncogenic probes were a kind gift procurred through Dr. A.Bamezai, Harvard Medical School.)

Antibody

(i) Monoclonal antibody Y-13-238 (Furth et al., 1982) against ras protein p21, was a kind gift from Dr. A.Bamezai.

(ii) Secondary antibodies (a) Rabbit anti-rat IgG conjugated to HRPO (b) Goat anti-human IgG conjugated to HRPO and (c) Goat anti-human IgG conjugated to FITC were a generous gift from Dr. Batra, National Institute of Immunology (N.I.I), New Delhi.

(iii) Goat anti-human IgG (N.I.I., New Delhi) was used for panning.
METHODS

3.1 MAINTENANCE AND PROLIFERATION OF BLOOM SYNDROME (BS) AND NORMAL (GA~B) B-LYMPHOBLASTOID CELL LINES

Epstein Barr Virus (EBV) immortalized Blooms syndrome B-lymphoblastoid cell line (GM-03403 E) procured from NIGMS, Camden, NJ, USA were maintained in RPMI-1640 medium supplemented with 15% Fetal Calf Serum (FCS). The medium was supplemented with 2mM Glutamine and the cell lines maintained at 37°C in a CO₂ incubator with a supply of 5% CO₂ / 95% air.

Preparation of RPMI 1640 Medium

Reagents

- RPMI dehydrated medium
- HEPES 25 mM
- Sodium bicarbonate 0.2%
- Penicillin 100 units/ml
- Streptomycin 100 μg/ml

Dehydrated RPMI-1640 powder medium was reconstituted with one litre autoclaved double distilled water under aseptic conditions. For buffering, Hepses and Sodium bicarbonate was added to it. Antibiotics, Penicillin and Streptomycin, were also added to it. pH was adjusted to 6.8-7.2 with 1N Hydrochloric acid (HCl) whenever required. Medium was filter sterilized through 0.22 μm nitrocellulose filter. Sterile medium was aliquoted and stored at 4°C.
This medium was supplemented with 15% FCS and 2mM Glutamine.

*Cryopreservation and Revival*

The cultures were cryopreserved in liquid nitrogen from time to time, using the freezing medium consisting of 90% FCS and 10% cryoprotective agent, Dimethylsulfoxide (DMSO). Alternatively, freezing medium composed of 65% RPMI-1640 medium, 30% FCS and 5% DMSO also gave good recovery of live cells. Healthy cells were counted before cryopreservation, by trypan blue exclusion method, pelleted and suspended in 1 ml of the chilled freezing medium in a freezing vial and kept overnight at -70°C, before preserving in liquid nitrogen. At the time of freezing, the cells were 90% viable and the whole process before freezing the cells was carried out at 4°C.

For recovery of frozen cells, the vials were removed from liquid nitrogen tank and immediately incubated in a waterbath maintained at 37°C. Immediately after thawing, the cells were washed three times with RPMI-1640 medium supplemented with serum and then transferred to a fresh culture flask. These cultures were then allowed to proliferate in 5% CO₂ atmosphere. Recovery of live cells on revival varied from 50-75%.

3.2 NEoplast ic TRANSFORMATION IN VITRO

3.2.1 Dose-Selection of Carcinogen

The dose of the carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), was selected by performing a viability count by Trypan blue exclusion technique using
a hemocytometer. Aliquots with 2 x 10^6 cells were exposed to different concentrations of the carcinogen for 24 hours. The doses used were 0.03 μg/ml, 0.3 μg/ml, 0.5 μg/ml, 1 μg/ml and 10 μg/ml. The concentration at which both the BS and GA_3 cells showed less than 50% cell death after 24 hrs of MNNG exposure was chosen as the dose for transforming the BS cells.

Having determined the dose, 10 x 10^6 cells from Bloom syndrome (BS) as well as normal (GA_3) B-lymphoblastoid cell lines were exposed to MNNG. After 24 hours of exposure the cells were washed twice with RPMI-1640 medium and once with serum supplemented medium. The cells were then reincubated in fresh RPMI medium at 37°C in a 5% CO_2/95% air environment. To determine the relative cell growth the cells were counted regularly for the first seven days followed by the counts at an interval of seven days for next twenty one days. The counting was done till the cells showed recovery in growth. The relative cell growth (N_t/N_0) was calculated where:

\[ N_t = \text{the number of viable cells on different days and} \]
\[ N_0 = \text{the number of viable cells on day 0.} \]

3.2.2 Soft Agar Assay

Soft agar assays were performed to check for malignant transformation. A base layer of 7 ml of RPMI 1640 medium containing 20% fetal calf serum and 0.5% agar (Bactoagar, Difco laboratories) that had been extensively washed with distilled water to remove toxicity, was placed in the bottom of a 60-mm petridish. After the agar medium solidified, 3ml of a "seed layer" that consisted of RPMI 1640 medium, 0.4%
agar and 1 x 10^4 singly dispersed cells from Bloom syndrome (BS) or normal (GA3) B-lymphoblastoid cell lines (used as control) which had been exposed to MNNG for 24 hours, washed, and cultured for a month in serum supplemented RPMI 1640 medium, was placed over the base layer. The cultures were observed under an inverted microscope for single and even distribution of cells before being incubated for 3-4 weeks at 37°C in an incubator with a continuous flow of 5% CO₂. At regular intervals the plates were examined under phase contrast microscope to follow the growth of the colonies.

3.2.3 Nude Mouse Tumor Formation

Reagents

Phosphate Buffered Saline (PBS)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>0.144 M</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>2.7 mM</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>di-Sodium hydrogen phosphate</td>
<td>8.1 mM</td>
</tr>
<tr>
<td></td>
<td>pH 7.2-7.4</td>
</tr>
</tbody>
</table>

10 x 10^6 BS cells exposed to MNNG (BS-MNNG) for 24 hrs, and allowed to grow for 30 days in culture after removal of the carcinogen were pelleted and suspended in 100 μl of sterile PBS. This suspension was injected subcutaneously in the abdominal region of 6 week old male nude mice (BIO.LP). Cells from control cell lines, BS (unexposed to MNNG) and GA3 (exposed to MNNG) were also injected in nude mice (three for each cell line). The mice were monitored over a period of 6-10 weeks for tumor formation.
3.3 MORPHOLOGICAL AND CYTOGENETIC ANALYSIS OF MNNG EXPOSED BLOOM SYNDROME (BS-MNNG) CELLS

3.3.1 Scanning Electron Microscopy (SEM) (McCarthy et al., 1984).

Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>pH 7.2-7.3</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>2 % in PBS</td>
</tr>
</tbody>
</table>

2x10^6 BS and BS-MNNG cells were washed with PBS and incubated for 30 min in Glutaraldehyde at RT. These cells were further processed for Scanning Electron Microscopy at A.I.I.M.S, New Delhi. After the complete processing according to McCarthy’s protocol, the cells were viewed on a Scanning Electron microscope at A.I.I.M.S, New Delhi and photographs taken.

3.3.2 Chromosomal Aberrations and Sister Chromatid Exchange (SCE)

Chromosome aberrations and sister chromatid exchanges (SCEs) were studied both in MNNG exposed and unexposed BS and normal (GA_3) cells.

Chromosome preparation

Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colchicine</td>
<td>0.05 μg/ml</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.75 M</td>
</tr>
</tbody>
</table>
Methanol: Acetic Acid 3:1
Giemsa stain 5%

A minimum of $2 \times 10^6$ cells from growing cultures were exposed to Colchicine for 2.5 hours before harvesting the cells for chromosome preparation. The cells were pelleted at 1000 r.p.m and the medium removed. The cell pellet was suspended in 8 ml of KCl and incubated at 37°C for 8 min. The cells were again pelleted at 800 r.p.m and fixed for 10 min. in ice cold methanol : acetic acid :: 3:1. The chromosomes were spread on slides by air drying and stained with Giemsa. Chromosomal aberrations were scored according to the criteria laid down by ISCN (1985).

**SCE analysis**

**Reagents**

- Bromodeoxyuridine (BrdU) 10 μg/ml
- Hoechst 33258 0.05 μg/ml
- 2 X SSC
- Giemsa

A minimum of $2 \times 10^6$ cells were grown in the presence of 10 μg/ml of BrdU for 48 hours and harvested after exposure to colchicine for 2.5 hrs. Flourescence plus giemsa (FPG) technique was employed for differential staining of sister chromatids. The chromosome slides were incubated in dark at RT for 20 min. with the fluorescent dye, Hoechst 33258, rinsed in water and completely covered under a coverslip with 2 X SSC. These slides were then exposed to direct sunlight for 90 min., rinsed in water, stained with Giemsa and SCEs scored.
3.4 IMMUNOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF ANTIGEN BEARING BLOOM SYNDROME CELLS

3.4.1 Immunocytochemistry

To examine the carcinogen exposed and unexposed BS as well as normal cells for ‘ras’ gene product expression, immunocytochemical staining was carried out, using the Peroxidase-Antiperoxidase technique (Yoshida et al., 1988).

**Reagents**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration/Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>pH 7.2-7.4</td>
</tr>
<tr>
<td>Acetone</td>
<td>25%, 50%, 75%, 90%, 100%</td>
</tr>
<tr>
<td>Tris</td>
<td>1 M, pH 7.6</td>
</tr>
<tr>
<td>Diaminobenzidine (DAB)</td>
<td>10 μg/ml - diluted in 50 mM Tris, pH 7.6</td>
</tr>
<tr>
<td>Hydrogen Peroxide</td>
<td>3% in methanol</td>
</tr>
<tr>
<td>Meyer’s hematoxylin stain</td>
<td></td>
</tr>
<tr>
<td>Glycerine</td>
<td>90% diluted in PBS</td>
</tr>
</tbody>
</table>

A drop of cell suspension on glass slides was allowed to air dry and fixed by immersing in 100% acetone for 10 min. The fixed cells were hydrated by immersing the slides in a descending series (90%, 75%, 50%, 25%) of acetone concentration. The slides were rinsed in Tris buffer (0.05 M, pH 7.6) followed by incubation in 3% hydrogen peroxide at RT for 10 min., to eliminate endogenous peroxidase activity. Non-specific binding sites were blocked by incubating the slides at RT for 30 min. with goat serum diluted 1:20 with Tris buffer (0.05 M; pH 7.6). The slides were then washed in 0.05 M Tris buffer and incubated at RT for 60 min. with anti-p21 'ras'
monoclonal antibody (50 μl, 1:1000 dilution). The slides were washed thrice in Tris buffer and incubated at RT with HRPO (Horse radish peroxidase) conjugated anti-rat IgG (1:500) for 30 min.. After washing with Tris buffer, the slides were treated with freshly prepared substrate DAB / H₂O₂ (1 μl H₂O₂/ml of DAB solution) for 20 min., washed with distilled water, counterstained with Meyer's hematoxylin stain and mounted with coverslips after putting a drop of 90 % glycerine.

3.4.2 Detection of Cancer Associated Antigens

Serum from various cancer patients (already mentioned) was used as a source of primary antibody for screening the MNNG - exposed BS cells for the presence of cancer associated antigen. Normal cells (GA₃) and sera samples from healthy individuals were used as a control.

Serum separation and purification of IgG

Non-heparanised blood samples collected from cancer patients as well as healthy individuals were incubated for 1 hour at 37°C immediately after collection. Rimming of the clot with a needle was done and the samples left overnight at 4°C. The serum was carefully separated so that hemolysis did not take place and the clot was discarded.

Purification of IgG

Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulfate</td>
<td>&lt; 50%</td>
</tr>
<tr>
<td>Tris buffer</td>
<td>10mM, pH - 8.5</td>
</tr>
</tbody>
</table>
Sodium chloride

PBS

50mM, 100mM, 200 mM
(prepared in Tris buffer)
as described earlier

The sera samples were processed to obtain purified antibodies utilizing the methodology of Harlow and Lane (1988). The serum was precipitated with solid Ammonium sulphate with the maximum concentration of the salt not exceeding 50% (Table 4). The precipitate was collected by centrifuging at 10,000 rpm for 30 min at 4°C. This precipitate was dissolved in 10 mM Tris buffer and dialysed against three changes of the same buffer in order to remove the salt. The samples were loaded onto a DEAE-52 column which was extensively washed with 20 volumes of 10 mM Tris buffer (pH 8.5) prior to loading. The dialysed samples were passed through the column and the matrix bound antibodies were eluted with increasing Sodium chloride concentrations (50 mM, 100 mM, 200 mM). The fractions obtained were quantitatively analysed for the presence of protein and fractions with high protein content were combined. This eluted product was then dialysed against 10 mM Tris buffer, reprecipitated with Ammonium sulfate, and the precipitate collected by centrifuging at 10,000 r.p.m for 15-min at 4°C. The pellet was dissolved in PBS and dialysed against 3 changes of PBS. The solution thus obtained was that of purified antibody and this was checked by running on a 10% SDS-PAGE gel which yielded polypeptides of 24 and 55 kDa (Fig.2). The dialysed antibodies were stored at 4°C till use.

**Immunofluorescence**

Indirect immunofluorescence was performed both with cells fixed on slides and live cells.
<table>
<thead>
<tr>
<th>Starting Concentration</th>
<th>10%</th>
<th>20%</th>
<th>25%</th>
<th>30%</th>
<th>35%</th>
<th>40%</th>
<th>45%</th>
<th>50%</th>
<th>55%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>56</td>
<td>114</td>
<td>144</td>
<td>176</td>
<td>209</td>
<td>243</td>
<td>277</td>
<td>313</td>
<td>351</td>
</tr>
<tr>
<td>10%</td>
<td>-</td>
<td>57</td>
<td>86</td>
<td>118</td>
<td>150</td>
<td>183</td>
<td>216</td>
<td>251</td>
<td>288</td>
</tr>
<tr>
<td>20%</td>
<td>-</td>
<td>-</td>
<td>29</td>
<td>59</td>
<td>91</td>
<td>123</td>
<td>155</td>
<td>189</td>
<td>225</td>
</tr>
<tr>
<td>25%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>30</td>
<td>61</td>
<td>93</td>
<td>125</td>
<td>158</td>
<td>193</td>
</tr>
<tr>
<td>30%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>30</td>
<td>62</td>
<td>94</td>
<td>127</td>
<td>162</td>
</tr>
</tbody>
</table>

Values given are the number of grams to be added to 1 litre of solution to change the ammonium sulfate concentration from the starting concentration to final concentration.
Fig. 2  Purified antibodies as checked on SDS-PAGE (10%). Immunoglobulins from Hodgkin's lymphoma serum (HLS-6) and Trigon cancer sera (TC) purified after passing through DEAE-52 column are represented in Lanes 1 and 4 respectively. Lanes 2 and 3 represent the antibodies after ammonium sulfate precipitation of these sera samples. Standard molecular weight markers (M) were used.
Reagents

- PBS
- Formaldehyde
- Bovine serum albumin (BSA)
- Glycerine

Cells were fixed on slides by incubating in 4% formaldehyde for 10 min. at 4°C. The slides with the fixed cells were washed twice in PBS (pH 7.2) and incubated at 4°C in 2% BSA for 90 min, to block nonspecific binding. These slides were then rinsed in PBS and incubated at 4°C for 2 hours with the sera antibodies (1:30 dilution in PBS containing 2% BSA). These were then washed thrice in PBS, followed by incubation of 1 hr with the secondary antibody (Goat anti-human IgG conjugated to FITC) diluted 1:40 in PBS containing BSA. Finally, the slides were washed with gentle shaking to remove the unbound antibody, mounted in 90% non-fluorescent glycerine, and observed for surface staining, under an immunofluorescence microscope.

The initial studies were performed with the purified antibody, but in the later studies polyclonal serum as a whole was used. A similar procedure was carried out for the live cells, with the complete reaction being carried out in a microfuge tube before fixing the cells.

3.4.3 Panning

Cell population bearing a specific cancer associated antigen was enriched by the panning procedure.
Reagents

PBS
Cancer patients serum
Goat anti-human IgG

The panning procedure based on the observations of Wysocki and Sato (1978) was adopted to isolate cells expressing the antigens reactive with antibodies present in the cancer patient's serum. 3 ml of goat anti-human IgG (100 μg/ml) was used to coat the polystyrene bacteriological dishes. These dishes were stored overnight at 4°C. The plates were decanted and washed three times with PBS by gentle swirling. In order to separate cells bearing antigen(s) that reacted with the antibodies of cancer patient's, 5 x 10^7 cells were washed twice with PBS and incubated at RT with 3ml of purified antibodies (1:30) for 60 minutes. These cells were then washed with PBS twice and resuspended in 3 ml of PBS. The cell suspension was poured onto petridishes previously coated with Goat anti-human IgG. Care was taken to avoid bubble formation. The plates were incubated on an even surface at RT for 90 min. The nonadherent cells were removed by swirling the dish and decanting the supernatant. Plates were washed by gently pouring 10 ml of PBS along the sidewalls of the dishes and then swirling, tilting, and decanting. The adherent cells were recovered by pouring 10 ml of PBS onto the plates and flushing the entire surface with a pasteur pipette. The cells (positively panned against Hodgkin's serum sample were referred as BS-MNG-HL) thus recovered were cultured in RPMI-1640 medium supplemented with 15% fetal calf serum (FCS) and used for further characterization.
3.4.4 Protein Profile and Western Blotting

Protein analysis was performed by subjecting whole cell lysates of normal (GA₃) cells and the enriched cell population (BS-MNNG-HL) carrying antigen(s) reactive with the sera antibodies from Hodgkin's patients, to Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) as well as 2-Dimensional gel electrophoresis (O'Farrel, 1975) followed by Western analyses.

(a) *Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), (Laemmli, 1970)*

**Reagents**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>29.2 %</td>
</tr>
<tr>
<td>bis acrylamide</td>
<td>0.8 %</td>
</tr>
<tr>
<td>Tris</td>
<td>1.5 M pH 8.8</td>
</tr>
<tr>
<td>Tris</td>
<td>0.5 M pH 6.8</td>
</tr>
<tr>
<td>SDS</td>
<td>10 %</td>
</tr>
<tr>
<td>Ammonium persulfate (APS)</td>
<td>10 %</td>
</tr>
<tr>
<td>TEMED</td>
<td>100 %</td>
</tr>
<tr>
<td>Sucrose</td>
<td>15 % (w/v)</td>
</tr>
<tr>
<td>2 X Sample buffer</td>
<td></td>
</tr>
<tr>
<td>Tris</td>
<td>1.5 % (w/v)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>20 %</td>
</tr>
<tr>
<td>Dissolved in 35 ml water and adjusted pH to 6.8</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>4 %</td>
</tr>
<tr>
<td>2 mercaptoethanol</td>
<td>10 %</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.002 % w/v</td>
</tr>
</tbody>
</table>
The final volume was made to 100ml with water and stored in aliquots at -20°C.

Urea Lysis Buffer
- Urea 0.5 M
- NP-40 2 %
- β-mercaptoethanol 5 %
- Ampholines (optional) 2 %
- (pI 3.5-9.5, 5-7)
- PMSF 0.2mM

Reservoir buffer
- Tris 0.025 M
- Glycine 0.192 M
- SDS 0.1 %
- Water Volume made up with water

Staining Solution
- Methanol 50 %
- Acetic acid 10 %
- Coomassie brilliant blue 0.25 %
- Water 39.75 %

Destaining Solution I
- Methanol 40 %
- Acetic acid 10 %
- Water 50 %

Destaining Solution II
- Acetic acid 7 %
<table>
<thead>
<tr>
<th>Gel mixture</th>
<th>Single percentage</th>
<th>Gradient gel</th>
<th>Stacking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide sol.</td>
<td>7%, 10%</td>
<td>5%</td>
<td>20%</td>
</tr>
<tr>
<td>Tris (pH 8.8)</td>
<td>0.375 M</td>
<td>0.375M</td>
<td>0.375M</td>
</tr>
<tr>
<td>Tris (pH 6.8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1 %</td>
<td>0.1 %</td>
<td>0.1 %</td>
</tr>
<tr>
<td>APS</td>
<td>0.05 %</td>
<td>0.03 %</td>
<td>0.03 %</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.05 %</td>
<td>0.03 %</td>
<td>0.03 %</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>-</td>
<td>15% (w/v)</td>
</tr>
</tbody>
</table>

A minimum of 25 x 10^6 cells were washed with PBS and lysed with 250 μl of the urea lysis buffer. The lysates were frozen and thawed thrice in the lysis buffer and centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected and stored at -80°C till used.

Bio Rad gel electrophoresis unit for SDS-PAGE was used for running the gels of the required percentages. Two glass plates were assembled to form a gel mould with the help of three spacers. Separating gel of the required percentage was poured into it and overlayed with water. In the case of gradient gels, a gradient mixer was used for pouring the separating gel. Once the separating gels had polymerized, 5% stacking gel was poured and a comb was fixed to form wells. The slab gel was then suspended into the reservoir tank containing the same reservoir buffer, both in the upper and the lower reservoir chambers. Protein extract (containing 200-300 μg total protein) mixed with an equal volume of 2 X sample buffer was loaded in duplicate.
into each well. High molecular weight markers, that had been incubated for 45 min at 37°C were loaded in one well. The gel was run at an initial voltage of 80 V and once the dye had crossed the stacking layer, the voltage was increased to 120 V. These proteins were then electrophoretically transferred to 0.45 μm nitrocellulose paper and Western blotting analysis was carried out.

(b) *Electrophoretic transfer of proteins (Towbin, 1984) and Western analysis*

**Reagents**

Transfer Buffer

- Tris base 0.025 M
- Glycine 0.192 M
- Methanol 20 %
- Ponceau-S 0.5 %

Once the proteins had been separated, a part of the gel was stained with Coomassie blue. The other part of the gel was equilibrated in chilled transfer buffer for an hour before it was transferred by aligning Whatmann sheets and nitrocellulose paper cut to the size of the gel, in a manner so that a sandwich was formed. The gel was in direct contact with the nitrocellulose on one side, while on the other side it faced the Whatmann sheets. This whole assembly was held in place between two plastic plates, and it was immersed in the transfer buffer. The transfer was carried out at 15 V for 16 hours.

Once the transfer was complete a part of the blot including the molecular weight marker was cut out and the efficiency of transfer checked by staining with...
0.5% Ponceau S stain for 2-3 min followed by destaining in distilled water. The unstained blots were further processed for Western analysis.

**Western analysis**

**Reagents**

Tris Buffered Saline (TBS)

- **Tris** 25 mM
- **Sodium chloride** 0.144 M
- **Potassium chloride** 2.7 mM

Blocking reagent

- **Milk powder** 5 % (w/v)
- **Tween 20** 0.05 %
- **Volume made to 100 ml with TBS.**
- **Tris** 1 M, pH 7.6

Substrate solution

- **Diaminobenzidine (DAB)** 0.6 mg/ml of 0.1 M
- **Tris, pH 7.6**
- **H₂O₂** 1 μl/ml of DAB solution

Membrane strips with transferred proteins were incubated at RT with the blocking reagent for 12-16 hours. This was followed by two rinses of 2 min each in TBS. Membrane strips containing similar samples were incubated at RT with 3 ml of polyclonal sera/purified sera samples, from various cancer patients and pooled normal samples, diluted in 1:10 ratio with TBS containing 0.05 % Tween 20. These strips were incubated at RT for 6 hours with constant shaking. At the end of the incubation,
the membranes were washed 4 x 15 min at RT with TBS. The membrane strips were then subjected to incubation of 90 min with the HRPO conjugated secondary antibody (goat anti-human IgG) (1:500), with constant shaking. The strips were washed 5 x 15 min. each in TBS and then rinsed once in 0.1 M Tris pH 7.6. These washed immunoblots were developed at RT by incubating them in the substrate solution and subjecting to constant shaking, until the bands became suitably dark. The reaction was stopped by rinsing the immunoblots in distilled water.

(c) **Two-dimensional (2-D) gel electrophoresis (O'Farrel, 1975)**

Using 2-D gel electrophoresis the proteins from GA₃, BS, and BS-MNNG-HL cells were first separated on the basis of net charge (by isoelectric focussing) followed by further resolution of the polypeptides on the basis of molecular weight (by SDS-PAGE).

**Reagents**

**Isoelectric focussing (IEF)**

**Sample buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>9.5 M</td>
</tr>
<tr>
<td>NP-40</td>
<td>2 %</td>
</tr>
<tr>
<td>Ampholines pH 5-7</td>
<td>2 %</td>
</tr>
<tr>
<td>pH 3-10</td>
<td>2 %</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>5 %</td>
</tr>
</tbody>
</table>

Volume was made up to 5 ml with deionised distilled water and 200 μl aliquots were frozen at -70°C.
Sample overlay buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>2.4 gm</td>
</tr>
<tr>
<td>Ampholines</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>pH 5-7</td>
<td>0.025 ml</td>
</tr>
<tr>
<td>pH 3-10</td>
<td></td>
</tr>
</tbody>
</table>

Urea was dissolved and total volume was made up to 5.0 ml using deionised distilled water. 0.200 μl aliquots were frozen at -70°C.

SDS Equilibration buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>62 mM</td>
</tr>
<tr>
<td>pH 6.8</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>2.0 %</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10 %</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>5 %</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>50 mg</td>
</tr>
</tbody>
</table>

Volume was made up to 100 ml with deionised water. Aliquots (10 ml) were frozen at -70°C until used.

Agar solution

Agar 1% in SDS equilibration buffer without Bromophenol blue

Cathode solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium hydroxide</td>
<td>0.02 M</td>
</tr>
</tbody>
</table>

Anode solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoric acid (H₃PO₄)</td>
<td>10 mM</td>
</tr>
</tbody>
</table>
Stock Acrylamide (for IEF)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>28.4 gm</td>
</tr>
<tr>
<td>bis Acrylamide</td>
<td>1.64 gm</td>
</tr>
</tbody>
</table>

Dissolved in 100 ml of deionized distilled water and filtered through whatmann paper.

Gel Mixture (IEF)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>5.5 gm</td>
</tr>
<tr>
<td>Acrylamide solution</td>
<td>1.33 ml</td>
</tr>
<tr>
<td>10 % NP-40</td>
<td>7.97 ml</td>
</tr>
</tbody>
</table>

Ampholines

<table>
<thead>
<tr>
<th>pH</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5-9.5</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>5-7</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>10 % APS</td>
<td>10 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>7 µl</td>
</tr>
</tbody>
</table>

IEF gels were prepared in glass tubes sealed at the bottom with parafilm. Care was taken to carry out all 2-D analyses using identical set of conditions for reproducibility of results. To avoid trapping air bubbles, a thin needle of the size of the tube was used for filling the tubes with the IEF gel mixture to the marked position. The gels were overlayed with a small amount of water and after 1-2 hours this overlay solution was replaced with 20 µl of sample buffer and the tubes filled with water, till further use. IEF was carried out using a Bio Rad 2-D gel apparatus. The tubes and the upper chamber were filled with 0.02 M NaOH and the lower chamber with 0.01 M H₃PO₄, respectively. The gels were first electrophoresed without samples according to the following schedule:

(i) 200 V for 15 minutes
(ii) 300 V for 30 minutes
(iii) 400 V for 30 minutes
After this pre-run, upper chamber buffer was removed and 300-400 μg protein in the sample buffer was layered on top of the IEF gels and overlayed with sample overlay buffer. IEF gels with only sample buffer were run along, for determining the pH gradient formed during the run. The IEF gels were electrophoresed for a total of 4800 V-h.

Once the electrophoresis was over the tube gels were carefully extruded from the tubes using the trigone tubing apparatus and each tube gel equilibrated for 2 hours at RT, in 5 ml of SDS equilibration buffer before loading onto the second dimension gels. Equilibrated gels were stored in the equilibration buffer at -70°C till further use.

**Measurement of the pH gradient**

The IEF gels, run for determining the pH gradient were cut into 10 equal pieces and placed in vials containing 10 ml deionised, degassed water. After 45 minutes the pH of water in each vial was determined.

**Second dimension**

Second dimensional gels were gradient gels as described previously. After polymerization of stacking gels, the IEF gels were placed carefully on top of the stacking gel. The gel was kept in place by using 1% agar overlay buffer. Electrophoresis was started after the agar solution had solidified.
The 2-D gels were electrophoretically transferred and Western analysis with pooled sera samples of Hodgkin’s lymphoma cases and normal individuals was carried out as already described for 1-D gels.

3.5 MOLECULAR ANALYSIS OF CANCER-ASSOCIATED-ANTIGEN EXPRESSING TRANSFORMED BLOOM SYNDROME B-LYMPHOBLASTOID CELLS

3.5.1 High Molecular Weight Genomic DNA Isolation from Cell lines

Reagents

Proteinase K Solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Chloride</td>
<td>150 mM</td>
</tr>
<tr>
<td>Tris</td>
<td>10 mM</td>
</tr>
<tr>
<td>Ethylene diamine tetra acetate</td>
<td>10 mM</td>
</tr>
<tr>
<td>(sodium salt)</td>
<td></td>
</tr>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>0.4%</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>1 mg/ml</td>
</tr>
</tbody>
</table>

Phosphate buffered saline (PBS)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>0.14 M</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>2.7 mM</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>8.1 mM</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>3 M, pH 5.2</td>
</tr>
</tbody>
</table>

25 x 10^6 panned cells, growing in RPMI-1640 were washed twice with PBS. One volume of packed cells was mixed with ten volumes of Proteinase K solution and
incubated at 65°C for 15 min. This cell suspension was incubated at 37°C overnight in a waterbath shaker. Phenol : Chloroform : Isoamylalcohol (24:24:1) extractions were carried out at 4000 rpm at room temperature to remove proteins. One extraction only with Chloroform : Isoamylalcohol was done to remove phenol. To the clear aqueous phase thus obtained, 1/10 volume of 3M sodium acetate and 2 volumes of absolute EtOH were added to precipitate the DNA. The precipitated DNA was washed with 70% ethanol to remove salts, dried and finally suspended in 100 μl sterile water.

High Molecular Weight Genomic DNA isolation from Human Blood

(Kunkel, 1977)

Reagents

Lysis Buffer

Sucrose 0.32 M
Magnesium chloride 5 mM
Tris buffer 0.01 M pH 8.0
Triton X 100 1%

Digestion buffer

Sodium chloride 100 mM
Ethylene diamine tetra acetic acid 25 mM
Sodium salt (EDTA)
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>0.5 %</td>
</tr>
<tr>
<td>Tris</td>
<td>10 mM</td>
</tr>
<tr>
<td>pH 8.0</td>
<td></td>
</tr>
<tr>
<td>Proteinase K solution</td>
<td>20 mg/ml</td>
</tr>
<tr>
<td>Phenol</td>
<td>equilibrated with 0.1 M</td>
</tr>
<tr>
<td>Tris, pH 8.0</td>
<td></td>
</tr>
<tr>
<td>Chloroform : Isoamylalcohol</td>
<td>24 : 1</td>
</tr>
<tr>
<td>Isopropanol</td>
<td></td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>3 M</td>
</tr>
<tr>
<td>pH 5.2</td>
<td></td>
</tr>
<tr>
<td>Ethanol (EtOH)</td>
<td></td>
</tr>
<tr>
<td>EtBr</td>
<td>10 mg/ml</td>
</tr>
</tbody>
</table>

5 ml of nonheparanised blood was added to 45 ml of lysis buffer and incubated on ice for 15 min. It was centrifuged at 2800 rpm for 20 min at 4°C. The pellet obtained was gently resuspended in 4.5 ml digestion buffer. To this homogeneous cell suspension Proteinase K solution at a final concentration of 100 μg/ml was added. This cell suspension was incubated in a waterbath shaker at 55°C, overnight. Deproteination was performed by extracting the cell suspension twice with an equal volume of Phenol : Chloroform : isoamyl alcohol (24:24:1) and twice with Chloroform : Isoamyl alcohol (24:1) at 4000 rpm for 20 min at RT. To the aqueous phase thus obtained 1/10 volume of 3M Sodium acetate was added and the DNA was precipitated by adding Isopropanol, one and a half times the total volume. DNA was washed with 70% EtOH, dried and dissolved in 100 μl of sterile water.
The DNA concentration was determined spectrophotometrically by measuring absorbance at 260 nm. DNA quality was checked by running an aliquot of the DNA sample on 0.8% agarose gel followed by EtBr staining and visualisation under UV illumination.

**Agarose gel electrophoresis**

**Reagents**

10 X TBE  
Tris base 80 mM  
Boric acid 40 mM  
EDTA 2 mM

Agarose 0.8% in 1 x TBE

6 X Loading dye  
Bromophenol blue 0.25 %  
Xylene Cyanol 0.25 %  
Glycerol 30 %

EtBr 10 mg/ml

Agarose gel mixture was poured into the gel casting tray fitted with a comb and was allowed to polymerize for 60 min. The comb was removed and DNA samples mixed with 1/10<sup>th</sup> volume of loading dye were loaded in the wells. The gel was run at 50-80 V in 1XTBE. The gel was stained in EtBr for 30 min and the DNA was visualised by UV illumination at 302 nm.
Restriction enzyme digestion

Reagents

Restriction Enzymes
- Eco RI
- Hind III
- Xho I
- Cla I
- Hpa II
- Msp I

10 X buffers
Nuclease free BSA

10-15 μg of high molecular weight DNA isolated from different cell lines as well as from the blood of normal individuals was digested with different restriction endonucleases in appropriate digestion buffers. 30-40 units of the restriction enzymes were used for each digestion in a total volume of 50 μl containing 1/10th its volume, of loading dye. Samples were incubated at 37°C for 16 hours and a 5 μl aliquot was then checked for the extent of digestion.

3.5.2 Transfer of DNA Fragments to the Nylon Membrane (Southern, 1975)

Reagents

10 X TBE
pH - 8.3

Denaturing solution
- Sodium chloride 1.5 M
- Sodium hydroxide 0.5 N
For Southern blotting, restricted DNA from each cell line was run overnight in 10 x 15 cm long, 0.8 % agarose gels, in 1 X TBE at 25 V. Molecular size markers, comprised of lambda bacteriophage DNA digested with Hind III, were run in one lane. Gels were stained with EtBr, and photographed along with molecular size markers, rinsed in water and incubated in the denaturing solution for 45 min with gentle shaking. The gels were then rinsed with water twice and neutralised by keeping in neutralization solution for 45 min, with gentle shaking. Simultaneously three 12 x 20 cm long Whatman sheets were kept on a glass plate with their ends dipped in 10 X SSC solution in a glass tray. The gels were then gently transferred on these sheets. Care was taken not to entrap air bubbles in between each layer. The Nylon membrane previously soaked in 2 X SSC was kept on top of the gel. Three Whatman sheets cut to the size of the gel and soaked in 2 X SSC were kept over the membrane. Precut dry blotting papers, of the size of the gel, were stacked above the Whatman sheets. A glass plate over which about 500 gm equivalent of weight was put for proper capillary transfer was kept over the stacked blotting sheets. The transfer was allowed to continue for 16-34 hours, after which the membrane was rinsed in 2 X SSC. The DNA on the membrane was UV cross linked in Stratalinker by exposing to UV radiation of 1200 J/sq.cm. The membranes were air dried and stored at 4°C in sealed polythene bags until used.
3.5.3 Non-Radioactive Digoxigenin Labelling and Detection

Non-radioactively labelled probes were used for the detection of genomic variations in the MNNG exposed BS cells positively panned against Hodgkin's lymphoma sera samples (BS-MNNG-HL) and unexposed Bloom syndrome (BS) cell lines as well as the normal (GA3) cell line. Following protocols were adopted to amplify the recombinant vectors with insert sequences, to excise the inserts and to label these with non radioactive digoxigenin molecules to be used as probes in Southern analysis.

Preparation of competent cells by Calcium chloride treatment (Maniatis et al., 1984)

Reagents
Luria broth (LB) medium
  Bactotryptone 5 gm
  Yeast extract 2.5 gm
  Sodium chloride 2.5 gm
  Distilled water 500 ml
  Autoclaved prior to use
Solid LB medium 2 % bactoagar in LB medium
Ampicillin 50 mg/ml
Calcium chloride hydrated 1 M
Glycerol

Selection plates-Solid LB medium supplemented with antibiotic marker, ampicillin (100 μg/ml) was poured in petriplates and allowed to solidify.

A single colony of E.coli, strain DH5 α, was inoculated in 10 ml of sterile LB under aseptic conditions. The bacterial culture was grown overnight at 37°C at
200 rpm. The culture was diluted 1:200 in 200 ml LB by adding 1 ml of the overnight grown culture in 1000 ml flask. The diluted culture was again incubated at 37°C at 200 rpm. The bacterial growth was arrested by chilling the culture at 4°C when absorbance at 600 rpm reached 0.4-0.6 (2-2.5 hrs approx.). Bacterial cells were harvested at 6000 rpm for 10 min at 4°C under aseptic conditions. 25 ml chilled 0.1 M Calcium chloride was added to the cell pellet per 50 ml culture. Bacterial cells were harvested again at 6000 rpm for 10 min at 0-1°C. The pellet was resuspended in 2 ml of 0.1 M chilled CaCl\textsubscript{2} and incubated on ice overnight. Chilled glycerol (0.5 ml) was added to the above cell suspension. This cell suspension of competent cells was aliquoted in chilled eppendorfs and used for transformation or immediately transferred to -70°C for long term storage.

Transformation

An aliquot (200 µl) of frozen competent cells was taken and thawed on ice. This aliquot was incubated with the foreign DNA on ice for 45 min. Heat shock was given at 42°C for 2 min. Cells were immediately transferred to ice after the heat shock and 800 µl of LB was added to it. This mixture was incubated at 37°C for 1 hr. From this mix, 100 µl was plated on the antibiotic selection plate, which was then incubated at 37°C overnight for 16-24 hrs. One of the transformed colonies was picked up and streaked on a selection plate. Cells from this plate were then used to inoculate broth cultures containing suitable concentration of the appropriate antibiotic to which the plasmid conferred resistance.
Proper controls were used in order to avoid contamination problems. No DNA was added to the negative control whereas PUC 18 plasmid was used as positive control to check the transformation efficiency.

(a) **Plasmid DNA isolation (Maniatis et al., 1984)**

**Reagents**

- **TE buffer**
  - Tris 25 mM pH 8.0
  - EDTA 10 mM pH 8.0
- **Alkaline SDS solution**
  - Sodium hydroxide 0.2 N
  - SDS 1 %
  - Sodium acetate 3 M pH 4.8
  - Sodium acetate 3 M pH 5.2
- **RNase A Buffer**
  - Sodium acetate 0.1 M pH 5.2
  - Tris 500 mM pH 8.0
  - RNase 100 μg/ml
  - Tris saturated Phenol pH 8.0
  - Chloroform : Isoamyl alcohol 24:1
  - Ethanol

100 ml of overnight grown bacterial cultures were harvested at 10,000 rpm for 10 min at 4°C. The bacterial pellet was resuspended in 10 ml of TE buffer. To this cell suspension, 20 ml of freshly prepared alkaline SDS was added, and the cell suspension was incubated on ice for 10-20 min with intermittent mixing. 15 ml of 3M sodium hydroxide (pH 4.8), was then added to the cell suspension, mixed and left on
ice for 15 min. The lysed cell suspension was then centrifuged at 12,000 rpm at 4°C for 15 min. 0.6 volume of isopropanol was added to the supernatant to precipitate the DNA. The precipitated DNA was harvested by centrifuging at 12,000 rpm for 20 min at RT. The DNA pellet was dried and incubated with 10 ml RNase A buffer at 37°C overnight. To remove proteins and degraded RNA, DNA solution was extracted twice with an equal volume of Tris saturated Phenol : Chloroform : Isoamylalcohol (24:24:1) and once with Chloroform : Isoamylalcohol (24:1) at 12,000 rpm for 15 min at 4°C. To the aqueous phase thus obtained, two volumes of ethanol were added and the DNA precipitated at -70°C. The precipitated DNA was pelleted by centrifugation at 12,000 rpm for 15 min. and the pellet was washed with 70 % ethanol. This DNA pellet was then dried and dissolved in sterile water and stored at 4°C. The DNA concentration was determined by spectrophotometric readings at 260 nm, and the quality of the DNA was checked by running an aliquot of the sample in 0.8% agarose mini gel. The DNA was visualised under UV illumination.

(b) **Elution of DNA fragments from low melting agarose gels**

**Reagents**

- 10 X TBE pH 8.3
- Tris saturated phenol pH 8.0
- Chloroform
- Lithium chloride 4 M
- Low Melting Point Agarose 1 %

Plasmid DNA carrying the required inserts were digested with appropriate restriction enzymes. The digested DNA was electrophoresed on 1 % low melting
agarose prepared in 1XTBE. Molecular size markers were run in a separate lane (Fig.3). The insert sequence was cut out of the gel under UV illumination, and the band was made agarose free by incubating the block at 65-70°C for 15-20 min. Once the agarose had liquified, equal volume of phenol was added and the extractions were done at RT at 12,000 rpm for 3-5 min. These extractions were repeated till the aqueous phase containing the insert DNA was free of agarose particles. The aqueous phase was then extracted once with Chloroform, at room temperature at 12,000 rpm for 3-5 min. Finally the clear aqueous phase was spun at 12,000 rpm to remove any agarose contamination. 1/10th volume of 4 M LiCl and ethanol were then added to the aqueous phase.

The DNA was precipitated at -20°C overnight, and the DNA centrifuged at 12,000 rpm for 15-20 min at 4°C. The pellet was washed once with ethanol at 12,000 rpm for 15-20 min at 4°C. Then the pellet was dried and dissolved in 20 μl of sterile water. An aliquot was run on a 0.8 % gel to estimate the quantity of the eluted DNA before proceeding for the non radioactive labelling of the DNA.

(c) **Labelling of eluted DNA fragment**

**Reagents**

Nonradioactive Digoxigenin DNA labelling and detection kit (Boehringer Mannheim, Germany).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithium Chloride</td>
<td>4 M</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.2 M pH 8.0</td>
</tr>
</tbody>
</table>
Fig. 3  Ethidium bromide (EtBr) stained 1% low melting agarose gels depicting the excised inserts from their respective clones, schematically represented on the top of each gel. Each clone was digested with specific restriction enzyme and run along with HindIII digested λ bacteriophage molecular size marker (Lane M) and uncut DNA.

A) pH60 a genomic clone was double digested with EcoRI (Lane 2) and HindIII (Lane 1) to excise genomic fragment of 0.8 kb representing TCR-J-γ. The uncut DNA was also run (Lane 3).

B) pCMV-Neo Bam an expression vector was restricted with BamHI (Lane 1) to obtain 1.8 kb fragment, complementary to p53 gene. Lane 2 shows uncut plasmid DNA.

C) pBR322 clone was double digested with EcoRI (Lane 2) and ClaI (Lane 1) to excise the insert of 1.8 kb representing 3’ c-myc gene.

D) F8 clone was restricted with EcoRI (Lane 1) to obtain 2.0 kb fragment complementary to c-myb gene. The uncut plasmid DNA is shown in Lane 2.
Fig. 3
The eluted DNA was labelled by random priming with Dig11- dUTP using the nonradioactive Dig labelling kit.

1 μg of Eluted DNA was taken in a microfuge tube and the DNA was denatured by incubating the tube at 95°C for 15 min. The denatured DNA was immediately chilled on ice for 5 min and the following contents were added to the tube:

<table>
<thead>
<tr>
<th>Hexanucleotide mixture</th>
<th>5 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>dNTP labelling mixture</td>
<td>5 μl</td>
</tr>
<tr>
<td>Klenow enzyme</td>
<td>2.5 μl (5 units)</td>
</tr>
<tr>
<td>Final volume</td>
<td>50 μl</td>
</tr>
</tbody>
</table>

Volume was made up to 47.5 μl with sterile water. The contents were centrifuged for 1 min and incubated at 37°C overnight. The reaction was stopped by adding 5 μl of 0.2 M EDTA solution, pH 8.0. The labelled DNA was precipitated by adding 6.25 μl of 4 M LiCl and 200 μl of prechilled ethanol and incubating at -70°C overnight. The labelled DNA was obtained by centrifuging at 12,000 rpm for 10 min. The pellet so obtained was washed with ice cold 70% ethanol and then air dried. The pellet was then dissolved in 50 μl of sterile water.

**Direct detection assay**

**Reagents**

<table>
<thead>
<tr>
<th>Labelled Control DNA</th>
<th>5 ng/μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>(provided in the kit)</td>
<td></td>
</tr>
</tbody>
</table>
Buffer 1 Maleic acid
Sodium chloride

0.1 M
0.15 M
pH 7.5

Blocking stock solution
Blocking reagent
(provided with the kit)

10 % in Buffer 1

Wash Buffer
Buffer 2
Buffer 3
Tris
Sodium chloride
100 mM
100 mM
pH 9.5

TE
Tris HCl
EDTA
10 mM
1 mM
pH 8.0

Colour substrate solution
NBT solution
X phosphate
Anti-DIG-alkaline phosphatase conjugate
75 mg/ml (45 µl)
50 mg/ml (35 µl)
in 10 ml Buffer 3
750 U/ml

The labelling efficiency was checked by direct detection assay. A dilution series of labelled control DNA and labelled test DNA were made. These were then spotted onto a nitrocellulose membrane and the membrane was UV crosslinked. Further, it was rinsed briefly in Buffer 1. This was followed by an incubation of 30 min in Buffer 2. The membrane was rinsed in Buffer 1 and then treated for 30 min
with anti DIG antibodies diluted 1:5000 in Buffer 1. The unbound antibody conjugate was removed by washing the membrane 3 x 15 min with Buffer 1. The membrane was equilibrated for 2 min with Buffer 3 and incubated in the dark with freshly prepared colour solution sealed in a plastic bag. Within a few minutes, the coloured precipitates started forming. Once the desired spots were detected, the reaction was stopped by washing the membrane for 5 min in TE. Intensities of the colour precipitates produced by the labelled test DNA were then compared with that produced by the labelled control DNA, for the same dilution.

(d) **Hybridization and detection**

**Reagents**

Hybridization Buffer

- 6 X SSC
- Formamide 50 % (v/v)
- Sodium dodecyl sulfate 0.02 % (w/v)
- N-lauryl sarcosine 0.1 % (w/v)
- Blocking Reagent 5 %

Wash Solution I

- 2 X SSC
- SDS 0.1 % (w/v)

Wash solution II

- 1 X SSC
- SDS 0.1 % (w/v)
- Buffer 1 as used in direct detection assay

Wash Buffer
To obtain maximum signal to noise ratio in the chemiluminescent protocol, concentration of digoxigenin labelled probes were optimized for Southern hybridizations (Sachdeva et al., 1995).

The Southern blots were prehybridized at 42°C overnight in hybridization buffer without the probe. The buffer was removed the next day and replaced with fresh buffer containing the probe at an optimized concentration of 50 ng/ml in the hybridization buffer. Hybridization was done for 16-24 hrs at 42°C, in a waterbath shaker. The blots were then washed 3 x 30 min in Wash solution I at RT followed by three washes in Wash solution II at 60°C. The blots were rinsed in Buffer 1 for 5 min and incubated in 1 % blocking solution for an hour. Antibody (1:5000) treatment of these blots was done for 30 min at RT. Unbound antibodies were removed by washing the blots thrice in Wash Buffer, each wash being of 30 min. Further the blots were equilibrated in Buffer 3 for 2 min before exposing them to the substrate solution CSPD for 10 min. Damp filters were sealed in polythene bag and preincubated at 37°C for a maximum period of six hours and exposed to X-ray film in the dark for 5-30 min. The film was then developed. After detecting the signals the Southern blots were stored in TE buffer till reused.
Deprobing

Reagents

Deprobing solution
- Formamide 50%
- Tris 50 mM
- pH 8.0
- SDS 1%
- Wash solution 2X SSC

The Southern blot was thoroughly rinsed in water and incubated at 70°C in deprobing solution for 2 hr to strip off the previously hybridized probe. The blot was briefly rinsed in water and then washed 3 x 30 min at RT. The blot was then prehybridized and then hybridized with different probes under optimal conditions. Rest of the procedure followed was as described previously.