Chapter 4

DISCUSSION
There are several prospective of studying carcinogenesis since carcinogenic transformation is a highly complex and multi-step process. It is known that the process involves several events at molecular level including mutations of different kinds, gene rearrangements, gene amplifications, altered gene expressions, etc. to name the most obvious. These events occur during initiation, promotion and progression phases (Pitot, 1986). The repair systems, inherent in living beings, can potentially alter all the molecular events listed above. Thus, they exercise great influence on the transformation process in both human and animal models (Tomelette & Pfeifer, 1994), thereby, adding further complexity. In addition, an ever increasing number of chemicals, many types of radiations, numerous error in the normal cellular processes governing life, large number of identified oncogenes, etc. have also been implicated as causes of cancer. A process so diverse as cancer, therefore, remains uncontrolled despite advancement of knowledge base and technology. It is becoming more obvious now than earlier that the fight has to continue. We have not been able to zero down on the exact unified mechanism(s) by which cellular transformations are initiated and maintained essentially due to this extreme diversity of possibilities and causes. It has also not been possible to design a drug to mitigate suffering of individuals with different types of cancers. If that had happened, cancer would have been one of the diseases of the past like cholera, plague, tuberculosis, etc. These diseases of the past caused rampant loss of human lives, today they stand almost completely eradicated from the face of earth.

Therefore, there is a growing agreement among scientists and medical professionals of recent time that while the search for exact mechanisms of cellular transformation should continue, one must focus on early detection of onset of cellular transformation in order to increase the clinical efficacy of the present medical protocols. It has been postulated that such an approach can effectively increase the cancer therapeutic index. The present medical technology and tests are able to detect cancer at a later stage when it is too late to do much to help the individual. It is, therefore, of utmost urgency to find suitable 'biomarkers', which can act as a general, convenient and easy tool for detection of different types of cancers (Cuzick, 1999; Levi, 1999). A large group of scientists across the globe are working on finding or developing a convenient and easy way to detect cancer at an early stage. By doing so even the present medical intervention protocols can reduce human cancer related mortality and morbidity by an estimated 35% (Levi, 1999). Presently there are several tests that are employed to establish existence of cancer; none is conclusive (Ladik and Forner, 1994). In other words, the biomarkers of cancers currently in use are only indicative. Some of them are also expensive and cumbersome involving use of sophisticated instruments and technological know-how. The existing technology is helping to some extent the human population in developed countries, which also have better medical infrastructure and smaller populations. In countries like India and other developing or under-developed countries the scenario is very different. Most, especially rural population, do not have access to good medical infrastructure and the population density is high. Keeping these realities in mind, this piece of work was designed as a step in the direction of identifying a suitable biomarker of cancer that should be simple, sensitive and easy to apply as a tool for early detection of cancer and for mass screening.

Molecular biomarkers have such potentials as the control of all metabolic activities lies in genes located on the genome or chromosomes. Abnormal functions of the
genes or loss of control of their activities mediate cellular transformation as is evident from the studies relating to oncogenes or tumor suppressor genes (Kahn and Graf, 1986). Direct assessment of such events is likely to serve as the best biomarker for cancer detection. However, again use of molecular probes to directly detect such abnormalities of gene(s) is highly complex requiring sophisticated laboratory set ups and manpower besides being very expensive. These limitations make it difficult to apply such approaches for cancer detection in developing or under-developed countries. Furthermore, the metabolic abnormalities emancipating from such changes in gene(s) manifest their influences on metabolic processes through their products, proteins or enzymes. The abnormal gene product is the main cause of cellular transformation since they exercise the control of metabolic functions. Thus, it would be more pertinent if the search for such abnormal metabolic agent is made which can be used as biomarker for early detection of cancer. With this in mind, the work embodied in this thesis took up the probing of abnormality of gene(s) on chromosomes by biochemical characterization of such gene products and the metabolic pathway(s) controlled by the product(s). In this, poly ADP-ribosylation (PAR) of cellular proteins, including chromosomal proteins (histones), was chosen because of compelling evidences of its relevance to cellular transformation (Althaus and Richter, 1987; Boulikas, 1990; Boulikas et al., 1990; Sharan et al., 1998).

With this in background, the work of this thesis was designed to monitor gene(s) on chromosomes at biochemical level in order to characterize the chromosomes/genome during cellular transformation induced by chemical carcinogens. PAR of cellular proteins (total proteins as well as chromosomal proteins) has been taken as the main biological end-point of this study. The main gene(s) regulating this important metabolic process is located on chromosome 14 in mouse (MGI, 2003), the model used for the investigation as detailed in § 1.2. Cancer being an in vivo event, the study used a protocol of exposing normal healthy mice to carcinogens for up to 4 weeks to monitor the events of transformation essentially during initiation stage of transformation. The usefulness of mouse as experimental animal for research relating to cancer is high and the results of such investigations can be easily extrapolable to human situation (Berns, 1999). As has been described earlier, initiation is an irreversible but very important stage of transformation where cells commit to transformation (Pitot, 1986; Dorr and Koeller, 1994). Two qualitatively different chemical carcinogens were chosen for the work. The target specific hepatocarcinogen, dimethylnitrosamine (DMN) is a potent carcinogen causing liver transformation (Magee and Barnes, 1956; Montessano and Barsch, 1976; Pariat and Sharan, 1995; 1998). The dose of DMN used in this study has been shown to actually cause cellular transformation in mice liver (Pariat and Sharan, 1995). A general suspected natural carcinogen, aqueous extract of betel nut (AEBN), was also taken for the study due to its relevance to a large section of population across the world, especially in the northeastern part of India (IARC, 1985; 1987; Sharan, 1996). It is to be noted that AEBN is not reported to have any specific target organ for transformation unlike DMN, which specifically causes liver cancer. Nonetheless, a very high population index of cancer among betel nut chewers makes it an important cancer-causing agent. Since, the study used PAR of proteins as the biological end-point, a known chemical inhibitor of this metabolic process, 3-aminobenzamide (3-AB) was also utilized in the study as positive control (Sims et al., 1982; Banasik and Ueda, 1994).
4.1. STRUCTURAL CHARACTERIZATION

Structural architecture and integrity of chromosomes or genomic DNA is critical (Hancock and Boulikas, 1982). The integrity of the superstructure of chromosome has direct bearing on the functionality of gene(s) located on them (Poirier et al., 1982; Aubin et al., 1983; Boulikas, 1987; Bellard et al., 1989; Nagao et al., 1991; Saikia et al., 1999). The structural integrity of the genomic DNA can be conveniently monitored by DNase I induced fragmentation of DNA (Margison and O’Conner, 1979; Schneeweiss et al., 2000). DNase I randomly cleaves DNA, which is directly proportional to accessibility of the DNA to DNase I (Bellard et al., 1989). Therefore, if the DNA is in a condensed state, access of DNase I to DNA is limited and vice-versa. Consequently, limited or extensive fragmentation of genomic DNA shall result in its condensed or relaxed states, respectively (Margison and O’Conner, 1979; Schneeweiss et al., 2000). This can be conveniently visualized after the DNase I fragmented genomic DNA is electrophoresed on an agarose gel. This approach has been utilized in the initial experiments to follow the influence of DMN and AEBN on mice during initial 4 weeks of exposure. Fig. 5 shows that the DMN enhanced the susceptibility of genomic DNA isolated from spleen cells of mice toward DNase I digestion. An increasing degradation of genomic DNA was observed as DMN exposure increased. The genomic DNA isolated from SC of mice exposed to DMN for 4 weeks (lane 4, Fig. 5) was virtually totally degraded by DNase I under the conditions of experiment used in this study (§ 2.7.). The genomic DNA is heterogenous as evident in lane C (Fig. 5). However, certain stretches of DNA in the genome of untreated mice are accessible to DNase I in normal case also. This is shown as the large genomic DNA got fragmented to pieces of DNA of approx. size range of 10 to 60 kDa (lane C, Fig. 5). However, upon exposure to DMN, the genomic DNA seemed to undergo relaxation starting from 1st week itself and the process continued till the end of our observation period (lanes 2 to 4, Fig. 5). Thus, there was a general and consistent pattern of increase in the degree of degradation of DNA. These observations indicate that genomic DNA underwent relaxation almost immediately following exposure to DMN in vivo. DNA has highly complex and super-coiled structure in the normal physiological condition (Hancock and Boulikas, 1982). In this conformation DNA is normally inaccessible or slightly accessible to various chemicals/agents and nucleases for interaction. This is very important in order to maintain the integrity of such vital macromolecules that govern the very existence of the cell or the living system. The normal status of the DNA is unchanged as long as any external agents like chemicals, radiations, etc. or internal agents such as the free radicals do not force it to do so.

This relaxation of genomic DNA caused by DMN, as revealed by the ease with which DNase I progressively fragmented it, seems to be enhanced when 3-AB was concurrently administered on mice chronically exposed to DMN in vivo. In Fig. 6, it is evident that there was further increase in the degree of DNase I digestion of genomic DNA isolated from spleen cells of mice exposed to DMN + 3-AB. The relaxation of the genome was maximal in the 3rd and 4th weeks of treatment. Comparing Fig. 5 (DMN alone) with Fig. 6 (DMN + 3-AB) it stands out that the genomic DNA of mice undergoes faster relaxation when DMN is administered concurrently with 3-AB. Virtually entire genome was fragmented to pieces significantly smaller than 8.2 kDa in the 4th week. It can, therefore, be concluded that DMN, in presence of 3-AB, caused faster relaxation of the DNA conformation.
The introduction of pulse-field gel electrophoresis (PFGE) has revolutionized the way to analyze large DNA pieces, hallmark of eukaryotes, to get additional insights. PFGE can resolve DNA pieces on agarose gel in the size range of 30-50 kbp to well over 10 Mbp (Schwartz and Cantor, 1984). This was also used in the investigation to further our understanding on the genomic DNA isolated from DMN and DMN + 3-AB groups of mice. Following DNase I fragmentation, the samples were loaded for PFGE. The CU lane (Fig. 8) shows two large sized bands of DNA. Exposure to DMN caused progressive degradation of both these bands, which was relatively stronger for the lower band (lanes CU, C, 1 & 2, Fig. 8). In the 4th week, the entire genome was reduced to very small pieces of DNA by DNase I, which looked like a smear (Fig. 8). The result is in conformance with the agarose gel electrophoresis result (Fig. 5) and suggests that the smaller sized fragment of genomic DNA was the initial target of DMN interaction.

A general carcinogen, aqueous extract of betel but (AEBN), was also used in this study to follow its effects on the structural characteristics of genomic DNA or total chromosomes. AEBN, like DMN, also affected the structural organization of genomic DNA of spleen cells of mice chronically exposed to AEBN (Fig. 7). However, its effect was moderate as compared to DMN. AEBN also caused relaxation and opening up of DNA as evident by progressive ease of fragmentation by DNase I. However, the intensity was relatively less. The accessibility of DNase I to DNA was noticeable from 3rd week onwards of AEBN exposure (lanes 3 & 4, Fig. 7).

In our experiments evidence has been recorded of DMN, either alone or in conjunction with 3-AB, or AEBN induced change in the superstructure of genomic DNA or chromatin (Figs. 5-8). The super-structure underwent relaxation in both the case with different intensities. Structural change in genome has serious consequences on the functionality of DNA (Hancock and Boulikas, 1982; Schneeweiss et al., 2000). A progressively relaxed state of genomic DNA is taken as indicative of consequent higher transcribing or active genes (Margison and O’Conner, 1979). It has been shown earlier that carcinogenesis is favored when genes shift to relatively higher state of transcription (active genes) (Saikia et al., 1999; Schneeweiss et al., 2000). The results presented in this thesis also show that DMN pushed genes to progressively active state (Figs. 5, 8) and in presence of 3-AB the effect of DMN was further potentiated (Fig. 6). Even upon exposure to AEBN the effect was similar albeit less (Fig. 7). The dose of DMN used in this investigation is reported to cause hepatocarcinogenesis (Pariat and Sharan, 1995; 1998; 2002) and in that situation the genomic DNA of liver was in a relaxed state (Pariat and Sharan, 1998; 2002). The results (Figs. 5-8) show that even spleen cell genomic DNA was moved into relaxed state under the influence of DMN. It is possible that several genes of spleen cells also, including proto-oncogenes(s), were activated. This may means that initiation of transformation might have been made even in spleen cells after DMN or AEBN exposure. Some reports in literature indicate that qualitatively different carcinogens may have some molecular commonality during initiation stage (Magee and Barnes, 1956; Pariat and Sharan, 1998; 2002). This seems logical since during initiation the activated carcinogen interacts with gene(s) to cause mutations of different kind (Gabridge and Legator, 1969; Guttenplan et al., 1976; Pegg et al., 1978). Both DMN and AEBN have been reported to be metabolically activated or changed from potential carcinogen to ultimate carcinogen in mice when administered in vivo
(Magee and Barnes, 1956; Guttenplan et al., 1976; Lijinsky, 1976; Ashby et al., 1979; Rao and Das, 1989; Sharan, 1996; Saikia et al., 1999). An earlier study has shown that AEBN, at comparative dose induced DNA strand breaks in mouse kidney cells in vitro and enhanced rate of cell division (Wary and Sharan, 1988). A relatively higher dose was found to be toxic to kidney cell in vitro (Wary and Sharan, 1988). Moreover it has also been shown by Ames test that mutation frequency was significantly enhanced by AEBN (Balachandran and Sharan, 1998). AEBN was also found to cause unscheduled DNA synthesis suggesting damages to genomic DNA (Sharan and Wary, 1992). Once they interact with gene(s) the normal cellular processes are likely to be altered.

4.2. BIOCHEMICAL CHARACTERIZATION

Further molecular characterization of the genome (e.g. structural alterations, gene activation, gene amplification, etc.) involves use of specific gene probes. The outcome of such investigation, though very useful, shall have serious limitations on its applied potential (e.g., for mass screening of population for early detection of cancer) due to more than one reason. To start with molecular probes shall be needed which are expensive and fragile limiting the affordability to a smaller segment of the population especially in poorer countries. In addition, skilled manpower and sophisticated laboratory set-up shall be necessary to perform the assay for population screening. Thus, its application will be difficult in ordinary medical clinics and primary health centers in countries like India. Therefore, biochemical approach was chosen which could overcome practically all difficulties listed above. It is expected that the applied potential of the biochemical approach shall be significantly high even in developing and under-developed countries.

For the biochemical characterization, the biological end-point of status of poly ADP-ribosylation (PAR) of cellular proteins was chosen for many obvious reasons. Correlations between the cellular PAR and different cellular and molecular events including carcinogenesis have been observed in our laboratory and elsewhere (Benjamin and Gill, 1980; Althaus et al., 1995; Bhatia et al., 1990; Alderson, 1990; Moss and Vaughan, 1990; Boulikas, 1991; 1993; Saikia, 1996; Saikia et al., 1998; 1999; Pariat and Sharan, 1998; 2002; Devi, 2001). This post-translational modification of mainly chromosomal proteins, unlike other known post-translational modification, causes drastic changes in protein-DNA interaction essential to structural integrity of chromosomes (Hancock and Boulikas, 1982; Althaus, 1992; Aubin et al., 1983; Boulikas, 1991; Schneeweiss et al., 1995; Sharan et al., 1996; 1998a). Since indication of significant structural changes has been recorded in mouse genome following in vivo exposure of mice to DMN and AEBN (Figs. 5-8), it is prudent that we choose PAR as the biological end-point. Essentially, monitoring PAR of cellular proteins indirectly measures the activities of the two main genes controlling cellular PAR reaction. One of the two genes, poly ADP-ribose polymerase (PARP) gene, has been mapped on chromosome 14 of mice (Fig. 2) while the other, poly ADP-ribosyl glycohydrolase (PARG) gene, is proposed to be on the same chromosome. Their gene products catalyze PAR and the level of PAR is outcome of activities of these gene products (Althaus et al., 1995; Sharan et al., 1996). PAR was monitored by ELISA based immuno-probe assay, developed in our laboratory (Sharan et al., 1998b). This assay was chosen because of its sensitivity, reproducibility and the ease with which it could be used (Sharan et al., 1998b). Unlike the commonly used radioactive method
(Schneeweiss et al., 1995), the immuno-probe assay was totally safe from possible ill effects of radiation. The assay involved use of rabbit polyclonal antibodies (Ab) raised in the laboratory, the 1st Ab, against heterogeneous ADP-ribose polymer isolated from normal (control) mouse spleen cells (§ 2.11.). Commercially available anti-rabbit IgG tagged with alkaline phosphatase (ALP) was used as the 2nd Ab. Since the 1st Ab was raised against heterogeneous ADP-ribose polymers and not against any particular mouse proteins, the Ab worked as a general probe for all poly ADP-ribosylated cellular proteins. This gives a tremendous advantage to the assay employed in this work since any cellular protein carrying ADP-ribose polymer as a post-translational modification on it could be detected. The ALP conjugated to the 2nd Ab helped developed a distinct purple coloration of protein bands on slot- or Western blot. This could be easily quantified either by densitometry or by digital photography using appropriate software.

The obvious first task was to determine the immunogenicity of the 1st Ab and its specificity to detect ADP-ribose polymers on any protein. The immunogenicity was tested by Ouchterlony immunodiffusion assay (§ 2.12.). Fig. 9 shows a characteristic precipitin line appearing only between the wells containing the isolated spleen histone proteins (the Ag) and the 1st Ab. No precipitin line developed between BSA and Ab confirming the immunogenicity of the 1st Ab used for the investigation and also its specificity. The specificity of the 1st Ab against poly ADP-ribose was confirmed using SVP (§ 2.13.). As SVP very exclusively degrades ADP-ribose polymer from any protein (Sugimura, 1974), in principle, no detection of PAR should be made when a protein is totally free of ADP-ribose polymer. As is evident in Fig. 10 the color intensity of slots of histone proteins isolated from mouse blood lymphocytes was progressively diminished with increasing time of SVP digestion of the histone proteins before slotting (lanes 0-3, Fig. 10). There was no detectable color band after 30 min of SVP digestion of histones (lanes 4-6, Fig. 10). Equal amounts of histone proteins were loaded on each slot. Thus, these results (Figs. 9, 10) confirm that the 1st Ab used in the investigation was highly immunogenic and very specific to ADP-ribose polymers.

Using the immuno-probe assay, status of total PAR of all cellular proteins as well as individual, mainly histone, proteins were monitored for up to 4 weeks in diverse tissues (liver, spleen cells (SC), bone marrow cells (BMC) and blood lymphocytes) of mice chronically exposed to a hepatocarcinogen, DMN, or a general, naturally occurring carcinogen, AEBN \textit{in vivo}. DMN is a nitroso derivative of aliphatic hydrocarbons and has been shown to induce hepatocarcinogenesis (Magee and Barnes, 1956; Pariat and Sharan, 1995). Similarly, strong association of AEBN with human carcinogenesis has been suspected (IARC, 1985; 1987; Sharan, 1996). It has also been shown earlier that initiation of carcinogenesis in mice generally occurs within 4 weeks or so (Pariat and Sharan, 1998; 2002). Therefore, the period of investigation in this piece of work was limited to 4 weeks. The choice of liver, SC, BMC and blood lymphocytes for monitoring PAR of proteins were made to test applicability of the assay in diverse tissues of mice with different physiological states. While liver is a solid, multicellular and pre-mitotically fixed tissue, SC, BMC and blood lymphocytes are not so. Further, SC and BMC are heterogeneous cell population of differentiating cells while blood lymphocytes are homogeneous differentiated cell population. Blood lymphocytes were chosen for the work for an additional important reason. From the viewpoint of possible applied use of results in
screening of population for early detection of cancer, blood lymphocyte is a very convenient tissue. Drawing of blood from a subject requires minimal medical intervention and causes virtually no trauma. If blood lymphocytes mirror biochemical and physiological status of a subject, use of biopsies could be totally avoided in the future. As we know, present medical practices heavily depend on analysis of biopsies especially in cancer diagnosis. There is not only surgical intervention and trauma involved in the process of obtaining biopsies; there are ethical questions too associated with it. Therefore, investigation using blood lymphocytes assumes further significance.

DMN progressively lowered the total PAR of cellular proteins in different tissues investigated. This lowering was seen on total PAR of cellular proteins (lanes II, Fig. 11) by slot-blot immuno-probe assay (§ 2.18. & 2.21.). This was observed while the total protein slotted remained essentially constant as evident by the ink stained (§ 2.22.) replica slots (lanes I, Fig. 11). Even the protein profile of these tissues did not show any noticeable visible changes (Fig. 29) suggesting that during the period of observation quantitative changes in cellular proteins did not occur. Table I - IV and Figs. 15 - 18 show the quantification results of total PAR of proteins in liver, SC, BMC and blood lymphocyte, respectively. The quantification data on total PAR (Tables I - IV) and plots (Figs. 15-18) confirm the visual impression emancipating from the immunoprobed slots (Fig. 11). In general, there was a near-linear but inverse relationship between the periods of DMN exposure and the total PAR of cellular proteins in all the tissues examined. The lowering was also statistically significant in most case, especially after 2 weeks of DMN exposure to mice in vivo (Tables I - IV). While liver, SC and BMC showed the maximum inhibition of total PAR, over 60 % in 4th week as compared to the control (Tables I - III), blood lymphocytes exhibited about 45 % inhibition during the same period (Table IV). The effect of AEBN, the other general carcinogen used in this investigation, was examined in SC, BMC and blood lymphocyte only (Fig. 14). Total PAR of cellular proteins also showed similar trends after quantification with tissue specific variations (Figs. 26 - 28) while the proteins slotted for immunoprobe assay (lanes I, Fig. 14) or the proteins profile (Fig. 32) were essentially invariant. Except in SC, the lowering of total PAR was statistically significant as compared to the respective controls (Tables XII - XIV). The effect of AEBN exposure on total PAR of BMC proteins was maximal in 4th week (reduced to nearly 75 % of the control) (Table XIII). The effects of AEBN exposure in SC (Table XII) and blood lymphocytes (Table XIV) were comparable to that in case of DMN (Tables II & IV). The results, therefore, suggest that both carcinogens, irrespective of their chemical differences or tissue specificities, lowered total PAR of cellular proteins, thereby inducing qualitative differences as exposure to the carcinogens progressed up to 4 weeks in vivo.

3-Aminobenzamide (3-AB) is a potent chemical inhibitor of PARP (Purnell and Whish, 1980a; Christine and Bernard, 1987; Banasik and Ueda, 1994). Therefore, it shall also exercise influence on the cellular PAR reactions (Rankin et al., 1989; Purnell and Whish, 1980a; Devi, 2001). To dwell further into the biochemical process, 3-AB was acutely administered to mice simultaneously with chronic, low-dose DMN exposure. Slots of samples from DMN + 3-AB exposed mice (Fig. 12) show expected further reduction in total PAR of cellular proteins. Quantification of the total PAR of proteins of liver (Table V; Fig. 19), SC (Table VI; Fig. 20), BMC (Table VII; Fig. 21) and blood lymphocytes (Table VIII; Fig. 22) make the point
obvious. With the exception of liver, SC, BMC and blood lymphocytes exhibited higher extent of inhibition of total PAR of cellular proteins following concurrent exposure of mice to DMN + 3-AB than only DMN. It is, however, not clear why liver, which is the target organ for DMN induced carcinogenesis, did not reproduce the trend (compare Tables I & V), rather showed in opposite. This point shall be investigated in the future. Nonetheless, the extent of lowering of total PAR of SC, BMC and blood lymphocyte proteins was further accentuated by the concurrent presence of 3-AB. In case of blood lymphocytes, which showed minimal lowering effect of DMN on total PAR of proteins compared to liver, SC and BMC, the combined exposure of DMN + 3-AB caused significantly more effect on PAR (compare Tables IV & VIII). The level of PAR at the 4th week of DMN administration (Table IV) went down from 54.41 ±6.53 to 28.39 ±2.32 (Table VIII). This is expected since the concentration of 3-AB used in this investigation (2 mM) is reported to inactivate PARP in vivo (Christine and Bernard, 1987; Devi, 2001). As has been recorded, DMN also caused lowering of total PAR of proteins. Thus, together, they are likely to cause higher order of lowering of total PAR of cellular proteins. This suggests that DMN, a known hepatocarcinogen, and 3-AB, a chemical inhibitor of PARP, work in the same direction as far as PAR reaction is concerned. From this viewpoint, chemical inhibition of PAR reaction is likely to create a similar biochemical situation in a living system to that by DMN. Together they will simply aggravate the situation further.

Since the main target proteins for PAR is reported to be chromosomal proteins (Althaus et al., 1995; Miwa and Sugimura, 1990; Sharan et al., 1998a; Pariat and Sharan, 1998; Devi, 2001; Kun et al., 2002), especially histone proteins (Boulikas, 1990; Schneeweiss et al., 1995; Sharan et al., 1996; 1998a; Pariat and Sharan, 1998), the effects of DMN and AEBN were also monitored on the total PAR of histone proteins isolated form liver, SC and blood lymphocytes of mice exposed to the carcinogens in vivo. The isolated histone proteins (§ 2.17.) were slot-blotted on NC membrane and immuno-probed. Since only histone proteins were slotted, the resulting PAR signal shall indicate the state of poly ADP-ribosylation of histone proteins exclusively. The result of slot-blot immuno-assay to measure total PAR of all histone proteins isolated from liver, SC and blood lymphocytes of mice exposed to DMN is shown in Fig. 13, lanes II while lane I shows the replica slot stained for total histone proteins by India ink. Their quantification data have been shown in Tables IX, X & XI, respectively. It is evident that the general trend of lowering of total PAR of cellular proteins with progression of DMN exposure period was essentially mirrored by the plot of total PAR of isolated histones vs. DMN exposure period (Figs. 23 – 25). The plot for liver (Fig. 23), however, showed some resistance to the lowering effect of DMN on the total PAR of histones in the 2nd and 3rd weeks of DMN exposure making the effect statistically non-significant (Table IX). Barring this, all reductions of total PAR of histone proteins in liver, SC and blood lymphocytes were statistically significant at p ≤ 0.05 and 0.01 as compared to their respective controls (Tables IX – XI). While this qualitative change was recorded on total histone proteins, there was no evidence of quantitative changes in histone proteins under the influence of DMN exposure. The profile of histone proteins isolated from liver, SC and blood lymphocytes exhibited no apparent differences between the controls and treated samples (Fig. 31). Thus, it can be concluded that DMN induced qualitative changes in histone proteins by way of reducing their extents of poly ADP-ribosylations during the initiation stage of DMN induced carcinogenesis.
To analyze the PAR of individual histones, the isolated histone protein mixture from liver, SC and BMC of mice exposed to DMN was electrophoresed by SDS-PAGE, Western blotted the resolved histone proteins on NC membrane and immuno-probed (panel I, Fig. 35). A replica blot was stained by India ink to visualize all proteins resolved. Different histone proteins have been identified (panel II, Fig. 35). It is evident from Fig. 35 that most histones of liver, SC or blood lymphocytes were poly ADP-ribosylated and showed similar and expected trend of lowering of PAR with period of DMN exposure. Quantitative plot of PAR for liver (Fig. 45), SC (Fig. 46) and blood lymphocytes (Fig. 47) reaffirm the observation. The isolated histone proteins have been identified and, for the purpose of quantitative analysis, grouped into three categories, namely H1, H3/H2b and H2a in this study. This was done based on their migration on PAGE gel and convenience of quantification (Fig. 35). While the PAR of histone H1 reduced to about 45 % of control in the 4th week of DMN treatment in liver (Tables XXXIV) and blood lymphocytes (Table XXXX), SC showed relatively more effect reaching nearly 32 % of the control (Table XXXVI). Histones H3/H2b also follows the same general pattern of diminution of PAR showing less effect in liver (Tables XXXV) and blood lymphocytes and more effect in SC (Table XXXVIII). Histone H2a also repeated the pattern exhibiting differential PAR in the three tissues examined (Tables XXXVI, XXXIX and XXXXII for liver, SC and blood lymphocyte, respectively). The plots of these data are given in Figs. 45, 46 and 47 for liver, SC and blood lymphocytes, respectively. The general conclusion that may be arrived at is that PAR differentially modifies different histone proteins in different tissues. This is in line with earlier reports of the same nature (Schneeweiss et al., 1995; Sharan et al., 1996). The histone proteins of SC seemed to be relatively more modified by PAR under the influence of DMN while the target organ for DMN induced transformation, liver, as well as blood lymphocytes showed lower extent of PAR of its histone proteins under the influence of DMN. There will be need of further investigation to clarify the reason for this differential effect of DMN.

By comparing the levels of total PAR of cellular proteins and that of total histone proteins, it is evident that the major target of cellular PAR are histone proteins. This is in line with earlier reports that almost 90 % of total cellular PAR is contributed by the PAR of different histone proteins (Boulikas, 1990; Boulikas et al., 1990; Schneeweiss et al., 1995; Sharan et al., 1998a; Pariat and Sharan, 1998; Devi, 2001), making histones the most preferred target proteins for PAR. Qualitatively different extents of PAR of histones shall influence its weak-interactions with DNA to organize the chromatin superstructure (Boulikas, 1991). Since it was shown that the chromatin underwent relaxation under similar conditions (Figs. 5, 8), it appears that lowering of total PAR of histone proteins may have facilitated the relaxation of the chromatin organization.

Considering the predominance of histone proteins in PAR reaction and consequent effect of this reaction on the structural/functional organization of chromatin, it was desirable to observe the PAR of individual cellular proteins in the whole homogenate (WH) of tissues obtained from mice exposed either to DMN or AEBN. To achieve this, cellular proteins in the WH of tissues were first resolved on 12 % SDS-PAGE gels (§ 2.19.). The resolved proteins on the gels were transferred onto NC membrane by Western blotting (§ 2.20.). The Western blot was immuno-probed with the 1st Ab followed by 2nd Ab and the bands containing poly ADP-ribosylated proteins were
color developed (§ 2.21.). As in case of slot blots, the protein bands on the Western blots were also quantified. A replica Western blot was simultaneously stained with India ink to ascertain the amounts of proteins loaded in each lane (§ 2.22.). The immuno-assay, being specific to poly ADP-ribose modification and not to any particular cellular protein, should detect all poly ADP-ribosylated cellular protein bands on a Western blot. In this, if histones are the preferred targets for PAR, one may expect that mainly histone proteins would be detected on a Western blot subjected to the immuno-assay. This was indeed observed.

The Western blots of WH of liver, SC, BMC and blood lymphocytes of mice exposed to DMN in vivo are shown in Fig. 33. Results of identical experiment for AEBN exposure are shown in Fig. 36. In both the cases, the left panel (I) has been immuno-probed while the right panel (II), a replica Western blot, was stained with India ink. As expected, there was no noticeable quantitative difference in the amount of proteins blotted for all four tissues examined as revealed by ink stained blots (panels II, Figs. 33 and 36). However, upon immuno-probing, the qualitative difference between individual proteins in terms of poly ADP-ribosylation was apparent as DMN (panels I, Fig. 33) or AEBN (panels I, Fig. 36) exposure periods progressed. It was again apparent that histone proteins were the major target proteins for PAR among the WH proteins of different tissues. Under the influence of AEBN, PAR of individual proteins immuno-probed on Western blots showed a slightly different picture in SC, BMC and blood lymphocytes (Fig. 36). While histone proteins still appeared to be the main target proteins for PAR, unlike DMN, the bands were not so distinct.

Few high molecular weight proteins also seemed to be poly ADP-ribosylated (Figs. 33 and 36). It is known that PARP, molecular weight range 90 – 120 kDa (Kameshita et al., 1984; Shizuta et al., 1985), is among other non-histone protein, which is also a favored target of PAR (Althaus and Richter, 1987; Adamietz, 1987). This has also been shown earlier in studies using radioactive assay using ^32P-NAD^+ and cell lines in vitro (Schneeweiss et al., 1995; Sharan et al., 1996; 1998a). This process of self-modification of PARP is called autoribosylation or automodification. Automodification is proposed to be a regulatory mechanism for regulation of PARP enzyme activity (Althaus and Richter, 1987; Adamietz, 1987; Althaus, 1992; Althaus et al., 1995). Increasing automodification renders PARP progressively inactive and vice versa. In AEBN exposed group, high molecular weight poly ADP-ribosylated proteins appeared more like diffused bands in all three tissues (panels I, Fig. 36). In addition, some other relatively low molecular weight cellular proteins were also modified. In this, an important and relevant class of non-histone proteins is high mobility group (HMG) proteins. The HMG proteins have also been reported to be modified by PAR under the influence of DMN (Pariat and Sharan, 1998; 2002). These are the protein bands besides histones and PARP that are visible on the Western blots after immuno-probing (panels I, Figs. 33 and 36). Western blots of isolated histone proteins from liver, SC and blood lymphocytes of mice exposed to DMN upon immuno-probing showed almost similar patterns and visual impression of lowering of PAR of histones with extension of DMN exposure period was obvious (Fig. 35). It has to be noted that isolated histone proteins resolve into different histones quite differently by SDS-PAGE when compared to resolution of cellular proteins from WH. Being highly basic and free from other cellular proteins, the profile of isolated histones looks different.
The concurrent presence of 3-AB with DMN, on expected lines, further significantly inhibited cellular PAR reaction as evident from the Western blots of WH of liver, SC, BMC and blood lymphocytes of mice exposed in vivo to DMN + 3-AB (Fig. 34). Lanes loaded with samples from 2nd week onwards of exposure showed rather faint PAR signals (panels I, Fig. 34) while the right replica panels II, showed that almost equal amount of proteins were loaded for Western blotting. This is in confirmation with the results shown earlier for the total PAR of cellular proteins wherein also the same was observed when 3-AB was simultaneously administered on mice along with DMN (Fig. 12).

Visual impression of immuno-probed Western blots of WH showed the expected pattern of progressive lowering of PAR of individual proteins with progression of DMN or AEBN exposure periods. For further detailed quantitative analysis, two to three protein bands of the immuno-probed Western blots showing PAR modifications of proteins in different tissues were quantified. Two PAR modified protein bands were chosen from the immuno-probed Western blot of liver WH of mice exposed to DMN for analysis (Fig. 33, B). Protein band 1 (b1; mol. wt. 68 kDa) and 2 (b2; mol. wt. < 7 kDa) upon PAR quantitative analysis showed generally significant decline in PAR modification with progression of DMN exposure period (Tables XV - XVI, Fig. 37). In the WH proteins of SC from DMN exposed mice (Fig. 33, A), protein bands 1 and 2 (b1 and b2, Fig. 38) showed clear progressive reduction in the level of PAR (Tables XVII and XVIII). These proteins have not been identified but protein band 1 (mol. wt. 20 kDa) and 2 (mol. wt. 14.3 kDa) are likely to be histones H1 (mol. wt. 21.1 kDa) and H2b (mol. wt. 14.5 kDa), respectively, due to molecular weight similarities. Both these proteins showed significant reduction in their PAR modification with increasing DMN exposure period. AEBN exposure affected PAR of these two protein bands even more (Tables, XXXIII and XXXIV, Fig. 48). In BMC from DMN exposed mice, PAR of protein band 1 (b1; mol. wt. 14.3 kDa), likely to be histone H2b (mol. wt. 14.5 kDa) and a small molecular weight protein (b2; mol. wt. < 7 kDa) showed significant reduction in their PAR (Tables XIX and XX, Fig. 39). Blood lymphocytes from DMN exposed mice showed similar results for protein bands 1 (b1; mol. wt. 43 kDa) and 2 (b2; mol. wt. 14.3 kDa), likely to be histone H2b (mol. wt. 14.5 kDa) (Tables XXI and XXII, Fig. 40). The WH proteins from SC (Tables XXXXIII - XXXXIV), BMC (Tables XXXXV - XXXXVI) and blood lymphocytes (Tables XXXXVII - XXXXIX) of mice exposed to AEBN also repeated the observations for selected protein bands (Figs. 48 - 50, respectively). Simultaneous administration of 3-AB with DMN again produced results on expected lines for selected protein bands that were quantified from liver (Tables XXIII – XXIV), SC (Tables XXV – XXVII), BMC (Tables XXVIII – XXX) and blood lymphocytes (Tables XXXI – XXXIII) as evident in Figs. 41 – 44, respectively. In all, different tissues of mice exposed to either DMN or AEBN for up to 4 weeks exhibited expected declining but differential PAR of different cellular proteins. These WH proteins undergoing PAR modification in different tissues are likely to include histones H1, H3/H2b, H2a, H4 and some other proteins as apparent from analysis done above.

Reduction in the extent of poly ADP-ribosylation of particularly histone proteins has serious biological consequences. The first impact of this would be change in the structural organization of DNA since the charge status of histones shall be altered. Histones form an integral part in normal organization of DNA to form chromatin. As
is evident from DNase I degradation of genomic DNA or chromatin, the superstructure underwent progressive relaxation (Figs. 5-8) coinciding with decrease in the extent of PAR modification of different, mainly histone, proteins. The alteration naturally shall have impact on the functional status of gene on chromosomes (Poirier et al., 1982; Aubin et al., 1983; Boulikas, 1987; Bellard et al., 1989; Nagao et al., 1991; Saikia et al., 1999; FaraoneMennella et al., 2000). The level of PAR that has been assayed and quantified in this investigation is the result of the interplay of two main gene products involved in PAR metabolism, PARP and PARG (Sharan et al., 1996). These genes are proposed to be located on chromosome 14 in mouse (Figs. 1 and 2; MGI, 2003). The total genome of mouse underwent progressive relaxation (Figs. 5-8). Thus, it can be proposed that at least selected segments of chromosome 14 had undergone alterations in a way that the expressions of PARP and PARG genes were altered. In general a lowering of PAR of cellular proteins was observed in all cases. This may be caused by (a) low expression of PARP gene or (b) less active or inhibited PARP enzyme activity. Alternately, when (a) expression of PARG genes is more or (b) the PARG enzyme is more active then also the same will happen. This investigation did not address to these issues because one major purpose of this investigation was to look into the applied potential of PAR immuno-assay. However, the total outcome of any or all of these possibilities translated into lowering of extent of PAR of cellular proteins. This provides a valuable support to application of the results of the investigation for welfare of human kind.

4.3. Significance of the lymphocyte proteins results in light of DMN and DMN + 3-AB treatment

Early detection of cancer continues to be a formidable challenge to the scientific community and search for a suitable biomarker for use in mass screening of population is of utmost importance in our fight against cancer (Doll and Feto, 1981; Pelomaki et al., 1993; Doll and Koeller, 1994; Cuzick, 1999; Levi, 1999; Srivastava, 1999). Constant efforts are being made worldwide to find an effective and reliable biomarker and a convenient assay for early detection of cancers, especially in its initiation stage, which is when a cell gets committed to transformation. A good biomarker must be one, which could be common to all cancers. This broadens the scope of use of this marker for detection of any cancer. Poly ADP-ribosylation (PAR) of proteins meets the criterion. A decade of research in our laboratory had set a firm foundation of PAR of cellular proteins as an eminently suitable biomarker for detection of cancer (Sharan et al., 1998b). As elaborated earlier in this thesis, this post-translational modification of cellular proteins alters the charge and conformational status of the modified protein. The reaction is reversible. The target proteins include several chromosomal proteins/enzymes, including histone proteins, relating to chromatin organization and its function. The structural organization and functional state of chromatin, therefore, is postulated to be strongly influenced by PAR. The hallmarks of carcinogenesis include molecular events associated with the functionality of genes. Altered gene expression, accelerated or diminished gene activities and mutations are some such events associated with carcinogenesis. Since, PAR of chromosomal proteins also alters the functionality of chromosomes, the association of PAR of chromosomal proteins and carcinogenesis has a sound basis. In the strategy adopted in this piece of work for early detection of cancer, the level of PAR modification of cellular proteins has been assessed. An almost linear but negative correlation has been found between PAR of cellular proteins and initiation of
carcinogenesis by DMN and AEBN in a mouse model. It is to be noted that the
immuno-probing assay was employed in the investigation to detect PAR of proteins
irrespective of on which proteins the modification has been affected.

Usually for detection of cancer a confirmatory test comes when the biopsies of
cancerous tissues are investigated. Obtaining biopsies involves several medical and
ethical issues and poses problems when a patient is subjected to surgical procedures to
obtain biopsies. Further, obtaining biopsies of some tissues (e.g., brain tumor) is a
serious medical intervention besides being traumatic to a patient. In this piece of work
embodied in the thesis this issue has been addressed to directly. Besides tissues like
liver, spleen cells (SC) and bone marrow cells (BMC), blood lymphocytes were used
for the investigation after the mice was exposed to DMN or AEBN in a chronic
exposure protocol in vivo. The aim was to find out if any correlation existed between
the level of PAR of blood lymphocyte proteins and initiation of cancer. Simultaneously, the same was tested in liver, SC and BMC which also served as
positive controls for each other. Obtaining blood from any patient and separating
blood lymphocytes are routine medical practices involving virtually no medical
intervention, post-procedure medical care or trauma to a patient. Both drawing of
blood from a patient and separating blood lymphocytes are again very routine matters
requiring very basic and minimal medical infrastructures. The working hypothesis
was that if a correlation was found between PAR of blood lymphocyte proteins and
carcinogenesis then, in future, medical screening might only involve drawing of blood
from a patient for screening. This also makes it possible to handle a large number of
patients at ordinary hospitals and primary health centers and even para-medics shall
be able to do this.

All tissues examined in this investigation have shown a negative, nearly straight line,
correlation between period of DMN or AEBN exposures and PAR of proteins from
different tissues. The most significant observation made in this study that has direct
implication on its use in medical practice is that even blood lymphocytes exhibited
significant reduction in PAR modification of cellular proteins with progression of
DMN or AEBN exposure periods. This was in line with the trend exhibited by liver,
SC and BMC. Both total PAR of cellular proteins and PAR of individual histone
proteins showed similar trends. Different tissues or proteins did show variability in
terms of PAR, but the general message was clear. Tissue specific variations were
found which is normally expected. Though obtaining liver, SC or BMC from mice is
no problem but the same is not true for human situation. The results from mouse
blood lymphocytes give convincing clues that the strategy can be easily extrapolated
to human situation. Further, immuno-probe assay of total PAR of cellular proteins is
relatively simple as compared to the assay of PAR of individual proteins. The former
involves slotting the sample directly on a NC membrane (slot-blot) and immuno-
probing while, the latter, requires electrophoresis, transblotting the proteins from gel
onto a NC membrane (Western blotting) and then immuno-probing. Since our results
show a very clear, reproducible and statistically significant correlation between total
PAR of blood lymphocyte proteins and initiation of carcinogenesis induced by two
different classes of chemical carcinogens, DMN and AEBN, (Figs. 11, 13 and 14), it
is logical to postulate that this slot-blot immuno-probe assay can be employed in
human situation also. It is postulated, based on the results presented in the thesis using
mouse model that this strategy shall apply to any type of human cancer as well.
The biochemicals required for the whole slot-blot immuno-probe assay can, in principle, be packaged into a kit making it very convenient for transport. Most of the constituents of this kit have good shelf-life and require ordinary refrigeration, etc. The 1st Ab is the critical constituent which actually detects the PAR modifications of proteins. In the investigation, the 1st Ab was raised against heterogeneous ADP-ribose polymer obtained from mouse SC. Preliminary investigation on human blood lymphocytes using the same 1st Ab against mouse ADP-ribose polymer shows that the 1st Ab was also able to detect PAR of proteins in human blood lymphocytes. This already supports the basic premise of application of the assay for detection of human cancer and for its use as a tool of mass screening. However, all procedures shall need to be standardized for assay involving human samples before final conclusion is drawn. Much remains to be done before it is applied to detect human cancer. Nonetheless, the work carried out and elaborated in the thesis takes us closer to the goal of making cancer detection simple, sensitive and reliable in the near future.