ROLE OF TRANSLOCATED PROMOTER REGION PROTEIN IN TRANSCRIPTIONAL REGULATION OF C-JUN

REVIEW OF LITERATURE
Cancer and research directions in Cancer Biology

Cancer remains a deadly threat despite the best efforts of science. Cancer affects people at all ages, even fetuses, but the risk for most of the types of cancer increases with age (Cancer Research UK, 2007). Cancer causes about 13% of all the human deaths (Cancer World Health Organization, 2006). According to the American Cancer Society statistics, 7.6 million people died from cancer in the world during 2007 (American Cancer Society, 2007).

The active research on the development of drugs against cancer actually started after the president Nixon declared "War on Cancer" in 1971. While progress against cancer continues each year, the challenge remains a daunting one, which includes dramatic improvement of our approaches to detection, diagnosis, treatment and prevention and henceforth the discovery of possible therapies. A slightly improved understanding of the intricacies in terms of the molecular mechanism(s) underlying the disease progression and diagnostics have led to a number of new and effective treatments for cancer. The present day treatments include surgery, radiation therapy, chemotherapy, hormone therapy and combined treatment modalities such as chemo-radiotherapy but all of them suffer from severe drawbacks and side effects. Therefore, the emphasis in the clinical cancer research shifted towards therapies derived from biotechnology research, such as immunotherapy and gene therapy. Important aspect of the basic research includes an understanding and characterization of the potential mechanism(s) underlying carcinogenesis, with regard to the types of genetic and epigenetic changes, associated with the cancer development. The Cancer Genome Project stated in 2004 that "the central aim of cancer research has been to identify the mutated genes (cancer causing genes) that are usually implicated in oncogenesis" (Futreal et al., 2004). The goal of oncogenomics is thus to identify new oncogenes or tumor suppressor genes that may provide an insight into the cancer diagnosis and development of newer targets for cancer therapies (Strausberg et al., 2004; Bild et al., 2006).
Nearly all the cancers, known so far, result from the abnormalities in the genetic material in the normal cells (Kinzler and Vogelstein, 2002). However, the causes and implications of these abnormalities in the genetic material still remain an unsolved mystery. These genetic defects can be induced by chemical carcinogens, or infectious agents. The genetic abnormalities in cancer cells are expected to be inherently present in all the cells as these are either acquired inherited or are acquired during errors in replication. Cancer is a multistep process of the sequential alterations in either of the two genes, the oncogenes, tumor-suppressor genes or another class of genes, the microRNA genes resulting in genetic abnormalities. The first class includes the genes that promote cancer and are termed as proto-oncogenes, which are activated in cancer cells. The overexpression of their gene product is responsible for giving cells an altogether new phenotype, like hyperactive growth and division, protection against programmed cell death, promotion of the establishment of cells beyond the normal tissue boundaries and enable rapidly proliferating cells to establish in diverse tissue environments. The other class includes tumor suppressor genes that are inactivated in cancer cells. The loss of tumor suppressor gene within the cancer cells results in the loss of normal functions, such as appropriate DNA replication, cell cycle control, orientation and adhesion within the tissues. Such cells also possess the ability to escape the protective cells of the immune system and thus favor their survivability within the host.

The genetic changes in the genes are usually somatic events, but a person is generally predisposed to heritable or familial cancer because of the germ-line mutations. However, a single genetic change is rarely sufficient for the development of a malignant tumor. A tumor is comprised of cells that are derived from the already transformed cells harboring number of secondary or tertiary genetic alterations. Thus, the tumor cells often possess cytogenetically unique cells (heterogenous population). This heterogeneity within a tumor contributes to the differences in the clinical implications and responses to treatment of tumors with similar diagnostic patterns. The presence of the progenitor cancer cells in a tumor apart from the initial clone and subclone represents a wide variety of cells possessing different genetic alterations and states of differentiation (Croce, 2008).
This varied population of cells within a tumor with different characteristics renders the clinical management of the disease difficult and rather impossible. The difficulty in the treatment arises due to the differences in the sensitivity of the broad spectrum of cells within a tumor mass to chemotherapy, radiotherapy, and other treatments. For these reasons, an understanding of the initiation of the cancer is of great clinical significance and should be a priority in the development of a rational treatment for the cancer.

Most commonly, apart from the mutations in the tumor suppressor genes, most tumor cells exhibit dysregulated proto-oncogene functions. Importantly, dominant protooncogenes have been implicated in cancer and are thus considered as the prime targets for the pre-clinical trial studies these days. Few of such genes include growth factors, growth factor receptors, cell cycle regulatory proteins, transcription factors etc. The potential of all these proteins as drug targets, except for the ones involved in the cell cycle regulation have been tested in vitro and have reached till the human clinical trials. One possible method of ablating a dominant proto-oncogene is by the use of transdominant molecules which inactivates the proto-oncogene products by the heterologous expression of the mutant proteins that inhibit the function of the native version of the proto-oncogene. This is also called as dominant negative mutation strategy. However, this approach suffers from certain limitations and drawbacks to be used as cancer therapeutics. Apart from this, the ability of certain gene products in cancer progression, which are involved in altering the phenotypic characteristics of an advancing tumor (angiogenesis, metastasis and the ones which are responsible for providing resistance to drugs/therapy) have also been thought for, but not much progress has been made in this direction. Alternatively, this can also be achieved by curtailing the overexpression of the dominant proto-oncogene by inhibiting its interaction with the regulatory proteins at its own promoter/regulatory DNA sequences. This is possible only when we aim to identify the regulatory regions and subsequently the proteins, responsible for its enhanced transcription. Thus, the need of the hour is to focus on the latter strategy and to first identify all the genes involved in cancer. The further approach should be to identify the
proteins that modulate the expression of several proto-oncogenes, implicated in the cancer progression.

Tumorigenesis is a result of the genetic damage which occurred in the parental cell and remained persistent (i.e. not correctable) in all the cells of subsequent generations derived from the same mutant parental cell. The genetic damage in the cancer cells can be of two types; dominant (proto-oncogene) and recessive (tumor suppressor). Proto-oncogenes code for proteins that help to regulate cell growth and differentiation and are often involved in signal transduction and execution of mitogenic signals, usually through their protein products. Examples of proto-oncogenes include jun, fos, ras, wnt, myc, erk, and trk. Of the many known oncogenes, almost all except for a few are derived from the normal cellular genes (that is why called as proto-oncogenes) whose products participate in normal cellular pathways. For example, the ras is a proto-oncogene that encodes an intracellular signal-transduction protein; however the mutant version of this gene, rasD which is derived from ras is a potential oncogene and the overexpression of the gene product results in excessive or uncontrolled cell growth. Because most of the proto-oncogenes are basic to animal life, they have been highly conserved over the evolution. A gain-of-function mutation is usually responsible for the conversion or activation of a proto-oncogene into an oncogene.

Three possible mechanisms have been proposed that can produce oncogenes from their corresponding cellular proto-oncogene counterparts. Firstly, the point mutations in a proto-oncogene results in a constitutively acting protein product, which can cause dysregulation and an unnecessarily increase in the overall protein pool within a cell. Also, the mutation in the oncogene might result in the production of a mutant protein with enhanced stability, thereby prolonging its existence and continued activity within the cell. The second mechanism includes the localized reduplication (also termed as "gene amplification") of a DNA segment that bears a proto-oncogene, which results in the overexpression of the encoded protein and lastly, the the third mechanism suggest the chromosomal translocations, that bring a growth-regulatory gene under the control of a strong promoter causing inappropriate expression of the gene placed under its control. Thus, all these abnormalities at the gene level lead to an increased expression of
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the proto-oncogenes in the wrong cell type and at wrong time. This type of aberration in a dividing stem cell of the bone marrow causes adult leukemia. An oncogene formed by the first mechanism encodes an oncoprotein that differs in its characteristics slightly from the normal protein (corresponding proto-oncogene) unlike the oncogenes generated by the latter two mechanisms. The oncogene products encoded from the latter mechanisms are identical to the normal proteins in their characteristics; but their oncogenic effect is mediated either due to their overexpression or because of their inappropriate expression in cells where they are not destined to be expressed. Thus, with this basic knowledge of the functioning of the oncogenes and their gene products within a tumorigenic cell, we can say conclusively that an oncogene is a gene that, when mutagenized or expressed at inappropriate location and at high levels within a cell, transforms a normal cell into a cancerous cell.

The cells undergo cell death usually mediated by a programmed form of cell death, but the activated oncogenes can direct the cells destined to undergo apoptosis, to survive and proliferate instead. Under the category of tumor suppressor genes, the p53 gene product is the most characterized one and is often found to be mutated in majority of the human tumors. The p53 gene was originally identified as a major nuclear antigen in transformed cells. Latter, the studies indicated that the p53 gene product functions as a tumor suppressor as the mutant version of the p53 protein exhibited altered cell growth suppressor effects when compared to the wild-type p53, and promoted rapid cell proliferation in the cancerous cell. Therefore, it is quite evident that most of the oncogenes or tumor suppressor genes actually require additional steps such as mutation, amplification, duplication, to cause cancer.

The viral infections are also the causative agents of oncogenic transformations. The disruption of a gene due to the integration of a DNA element originated from a virus or a retrovirus, results in the expression of the viral gene product in a cell and will remain stable in all the cells derived from the same population. The role of several viral oncogenes had been identified in human...
cancers. Many cancer drugs have been formulated which target these DNA sequences and their gene products.

**Facts about c-jun and its regulation**

Transcription factors are the proteins responsible for binding to DNA to either stimulate or inhibit the transcription of the associated genes. Many transcription factors require interaction with other proteins for their activity (Shaulian and Karin, 2001, Idem, 2002). The proteins involved in the transcriptional regulation facilitate cooperative interaction with the accessory proteins by the juxtaposition of their contact surfaces with each other, that are either part of or tightly bound to the DNA binding domain (Chinenov and Kerppola, 2001; Ramirez-Carrozzi and Kerppola, 2001a, 2001b, 2001c; Dlakic et al., 2001). Transcription factors are often members of multigene families that share common structural domains. These transcription factors are involved in responses of cells to extracellular signaling agents, including growth factors and cytokines. Many genes that are altered, and subsequently results in cancer, encode proteins that are transcription factors. One such gene encoding a transcription factor is the myc (originally identified in the avian myelocytomatosis virus). A disrupted human myc was found in hematopoietic neoplasia. The disruption of the myc locus in the neoplasia was a result of retroviral integration, transduction and chromosomal rearrangements. The three myc genes, myc, n-myc and l-myc have been shown to be involved in cancer. Similarly, the fos was also identified in the feline osteosarcoma virus. The fos product was found to interact with another protooncogene product, c-Jun (encoded by c-jun) to form a functional transcriptional regulatory complex. c-Jun is the cellular counterpart of the transforming protein of the chicken retrovirus ASV17. The c-Jun protein is a component of transcription factor AP-1 (Angel and Karin, 1991), encoded by the c-jun gene, which is the cellular homolog of the retroviral v-jun oncogene (Vogt, 1990). The fifteen years old discovery of the c-Jun/c-Fos complex as an active AP-1 complex, implicated in the induction of gene transcription in response to the external stimuli, generated a great deal of excitement at that time. The Jun family of cellular transcription factors comprises of three members: c-Jun, Jun B and Jun
D. Amongst the AP-1 proteins, c-Jun seems to be most important for stimulation of cell proliferation (Shaulian and Karin, 2002). The putative proto-oncogenic function of c-Jun is primarily due to its role at the receiving end of a signal transduction pathway that mediates trans-activation in the presence of phorbol esters and other tumor promoters (Angel et al., 1987a,b). In addition to phorbol ester tumor promoters, such as TPA, AP-1 transcription factors are also activated by diverse physiological and pathophysiological stimuli including growth factors, oncoproteins such as Src and Ras, proinflammatory cytokines and UV radiation (Angel and Karin, 1991). This provided evidence and an explanation for the tumor promotion, in response to the agents that can activate the downstream signalling pathways (Figure 1). This in turn stimulate the activity of transcription factors that regulate the expression of several downstream genes involved in cell proliferation and subsequently result in neoplastic transformation.

\[ \text{c-jun} \] also belongs to the family of immediate early genes (IEGs) which are activated transiently and rapidly in response to a wide variety of cellular and extracellular stimuli, thereby representing a “standing response mechanism”,

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**Figure 1:** AP-1: a central switch converting extracellular signals into genetic responses. Extracellular signals acting through upstream protein kinase cascades affect the activity of the transcription factor AP-1, a dimeric complex composed of members of the Jun, Fos and ATF protein families.
wherein the activation at the transcription level is observed, before the synthesis of new protein products (Goyette et al., 1983; Webster et al., 1994; Hasmall et al., 1997). Thus IEGs are distinct from "late response" genes, which can only be activated later following the synthesis of early response gene products. Thus IEGs have been termed as "gateway to the genomic response". Such types of mechanisms are usually followed either by viral regulatory proteins, which are synthesized in the host post viral infection, or during the synthesis of cellular proteins immediately following stimulation of a resting cell by the extracellular responsive agents/signals. About 40 of such cellular IEGs have been identified so far. The earliest responsive and the best characterized ones include c-fos, c-myc and c-jun, which were found to be homologous to retroviral oncogenes. Therefore, the IEGs can be defined as early regulators of cell growth and differentiation.

The transcription factor c-Jun, product of the c-jun, is a bZIP protein that binds to specific DNA sequence either as a homodimer or as a heterodimer with other bZIP proteins such as the Fos family members, of which c-Fos is the commonest interacting partner (Schaefer et al. 2001). This c-Jun/Fos interaction is well characterized and is known to be mediated via the leucine zipper motifs through which the c-Jun forms homodimers and heterodimers with Fos and other jun-related proteins, which together constitute the AP-1 transcription factor that binds to the TPA response elements (TREs). c-Jun, therefore has the potential to mediate transcriptional regulation in response to a variety of stimuli. The c-Jun of diverse species have nearly identical amino acid residues in their dimerizing/DNA-binding bZIP domains with fairly similar residues elsewhere in their sequence, suggesting that the DNA binding and dimerization domains of these proto-oncogene products within the species remained conserved throughout evolution.

In addition to the c-Jun and c-Fos, AP-1 transcription factors are composed of homo-and heterodimers of basic region-leucine zipper (bZIP) proteins that belong to the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) subfamilies, all of which recognize the AP-1 binding site or TPA response element (TRE) in the regulatory region of the AP-1 target genes (Angel and Karin,
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1991; Shaulian and Karin, 2002). These proteins also can heterodimerize with other transcription factors such as members of the ATF/CREB and Maf/Nrl subfamilies (Shaulian and Karin, 2002).

The c-Jun protein possesses distinct domains, trans-activation domain and the DNA binding and dimerization domain. The activity of the c-Jun is highly dependent on the post translational modifications which include phosphorylation. The c-Jun is activated predominantly by the mitogen activated protein kinase (MAPK) cascade. MAPK tyrosine and threonine phosphorylation at the final level of the cascade activates three MAPK family members, extracellular signal regulated protein kinase (ERK), c-Jun amino-terminal kinase (JNK), and the high osmolarity glycerol response kinase (p38 α, β, γ, δ). AP-1 can be activated by all three of the MAPK pathways (Denhardt, 1996). c-Jun is a nuclear substrate of JNK1 and its transactivation activity is augmented by amino (N)-terminal phosphorylation at serines 63 and 73 present in the trans-activation domain (Smeal et al., 1991; Derijard et al., 1994) (Figure 2). The kinases responsible for this modification are the Serine activated protein kinase (SAPK)/JNKs. These kinases function efficiently by interacting with a very high affinity to the delta domain of the c-Jun. Since, this region is deleted in v-Jun, v-Jun remains transcriptionally active even without phosphorylation. Thus, the binding of an inactive SAPK to c-Jun might represent a mechanism for inhibiting the function and the subsequent activation of this transcription factor. Once SAPK is activated, it can phosphorylate c-Jun and dissociate from the protein. Mutations in the phosphorylation sites prevents dissociation thereby resulting in inactive c-Jun. v-Jun, on the other hand, cannot bind the kinases and so is constitutively active (Black et al., 1994). The C-terminal phosphorylation sites lie proximal to the DNA binding domain, and their phosphorylation by the protein kinases (including GSK-3 and CK-II) prevents DNA binding. These sites are dephosphorylated in response to growth stimulation and most likely function to repress the activity of the transcription factor during resting phase. Since Jun protein is present in many types of resting cells, this on-off phosphorylation provides a mechanism for rapid induction of Jun function.
Role of c-Jun in tumor progression

Transcription factors play a crucial role in the regulation of cell behaviour by modulating gene expression profiles. Many genes that are altered resulting in cancer encode proteins that are transcription factors. It would seem obvious that a genetic alteration that disrupts the normal expression and regulation of a transcription factor could have profound implications on cellular activities. Regulation of gene expression is thus highly dependent on accurate protein-protein and protein-DNA interactions. Previous studies have described a dual role for the AP-1 family of transcription factor, c-Jun in altering the cellular fate, as it is involved in various aspects of cell growth, differentiation, proliferation and apoptosis. The diverse roles played by c-Jun contribute to the cell proliferation and survival in various cell types in which c-Jun has been transiently activated along with a weak stimulation of JNK. On the other hand, a strong and prolonged activation of JNK and c-Jun might result in apoptosis. These effects are generally cell type specific and the molecular mechanisms underlying these antonymous responses mediated by c-Jun remain not fully understood. The c-Jun activity in transformed cells is regulated by signalling cascades downstream of proto-oncogene products which include Ras and Raf. In addition, the pro-proliferative role and the survival promoting function for c-Jun has been described in various cancer models.
The molecular mechanisms that upregulate the c-Jun expression in various cancerous cells are not clearly understood. Therefore, the primary interest is to elucidate the molecular mechanisms behind altered expression of c-Jun in various carcinomas, in order to successfully design specific inhibitors that can be used during pathophysiological conditions.

It has been hypothesized that the chronic elevation of c-Jun's expression or its activity by tumor promoters might promote oncogenic transformation. However, a more clear and direct genetic evidence in favor of this hypothesis has been lacking. Unlike other mammalian proto-oncogenes, mutations in the \textit{c-jun} locus have not been found in human or murine cancers and overexpression of the normal c-Jun protein does not readily result in transformation of rodent fibroblasts (Shaulian and Karin, 2002). Therefore, these observations obviate the possibility of involvement of this proto-oncogene in carcinogenesis unlike the well studied role of \textit{myc}, \textit{ras} and \textit{raf} oncogenes in several cancers. Contradicting the earlier reports, recent evidences emerged that supported the function and involvement of c-Jun in several types of cancers and in promotion of tumorigenesis. Studies demonstrate that a targeted disruption of the \textit{c-jun} in mouse hepatocytes does not interfere with normal function, but prevents the emergence of hepatocellular carcinomas in response to a classical model of tumor initiation-tumor promotion (Eferl et al., 2003). It has been seen that a profound proliferation defect occurs in mouse fibroblast lacking c-Jun, which was primarily attributed to the dramatic extension of their G1 transition time (Schreiber et al., 1999). These cells, when exposed to UV radiation did not undergo apoptosis, exhibited premature senescence and failed to re-enter the cell cycle (Shaulian et al., 2000). On the contrary, absence of other potential protooncogenes like JunD or Fos, in mouse fibroblasts did not reveal any defect(s) which suggested that the JunD served as a negative regulator of cell proliferation (Shaulian and Karin, 2002). Interestingly, the proliferation defect in c-Jun-deficient cells has been attributed to elevated expression of p53 and its target gene p21\textit{waf1} (Schreiber et al., 1999 and Shaulian et al., 2000). It has been reported that the overexpression of c-Jun alone is not sufficient for transformation of rodent fibroblasts. However, when it is coexpressed with the oncogenic Ha-Ras (V12) protein, the c-Jun augmented Ras-
mediated cell transformation (Binétruy et al., 1991). This synergistic potential of c-Jun with Ras is not exhibited by JunB or JunD and is dependent on the N-terminal phosphorylation of c-Jun by Jun kinases (JNKs) (Smeal et al., 1991). Furthermore, mouse fibroblasts lacking c-Jun are refractory to transformation by oncogenes, such as Hα-ras or v-src (Johnson et al., 1996). Thus, there is considerable evidence that c-Jun is required for proper cell proliferation and for oncogenic transformation in cultured fibroblasts. However, the evidence for c-Jun's involvement in tumor formation in vivo is still not clear. c-Jun-deficient mice die at embryonic stages from massive apoptosis of hepatoblasts, erythroblasts and other cell types (Hilberg et al., 1993; Johnson et al., 1993). To overcome this problem, mice harboring a "floxed" c-jun allele that can be deleted in designated cell types upon expression of the Cre recombinase have been developed. Exploiting this system, it was observed that c-Jun expression is required for proper proliferation in postnatal hepatocytes, suggesting its direct role in normal cell growth (Behrens et al., 2002). Moreover, its deletion in hepatocytes does compromise the ability of these cells to enter the cell cycle and undergo rapid proliferation after partial hepatectomy (Behrens et al., 2002). Also, the transgenic mice in which the c-jun gene has been knocked out in hepatocytes revealed a role of c-Jun in liver carcinogenesis (Eferl et al., 2003; Maeda and Karin, 2003). The results demonstrated that liver tumor mass was dramatically reduced and survival rate was notably improved in mice devoid of c-Jun. These results point directly towards the role of c-Jun in the pathways leading to cancer initiation and progression. Eferl et al., (2003) have investigated the mechanism(s) accounting for the reduced tumorigenic effect, and found that apoptosis was increased in liver tumor cells originating from transgenic mice, whereas the proliferation of these cells was not reduced in comparison to tumor cells derived from normal mice. These results contradicted the earlier work from the same group that had shown that c-Jun is required for optimal hepatocyte proliferation (Behrens et al., 2002). Most likely, the tumors that developed in c-Jun deficient mice have found ways to achieve high rates of cell proliferation even in the absence of c-Jun. Therefore, to account for this anomaly, inducible Cre expression system was used to determine at which stage of liver tumor development, c-Jun was required. The results suggested that
c-Jun is required in the initiation stage of liver carcinogenesis and not during progression. Yet, in another experiment, deletion of c-Jun after tumor initiation resulted in reduced tumor incidence. Most likely, c-Jun is required for the survival of initiated cells and therefore is needed for tumor promoter action, whose major function is to assist the survival and expansion of the initiated cells at the later stages. As the role of c-Jun in DNA repair or the effect of its deletion on formation of mutagenic lesions has not been evaluated, it was difficult to conclude that c-Jun is required for tumor initiation per se.

The much recent evidence supporting the observed role of c-Jun in cancer progression and tumor initiation came from the study conducted by Zhang et al., (2007). Their results demonstrate that the ectopic overexpression of c-Jun in several breast cancer cell lines results in increased movement (ability to metastatize) and invasiveness, indicating that c-Jun may play an important role in the progression of breast cancer. These results not only prove that c-Jun forms a critical component of the carcinogenic mechanism but also suggest that c-Jun antagonists might serve as successful candidates in chemoprevention of liver cancer, a significant health problem in certain parts of the world.

Apart from the known potential of the JNK to phosphorylate the transactivation domain of c-Jun, the JNK pathway has also been shown to play a critical role in tumorigenesis of oral cancer (Aranbayeva et al., 2005). The well documented role of the AP-1 complex in regulating the expression of several genes involved in human tumorigenesis was also proven in the pancreatic ductal adenocarcinoma. It has been shown recently that the elevated levels of AP-1-binding activities and multiple AP-1/DNA complexes containing c-Jun, predominate in pancreatic cancer cells. Furthermore, the studies also demonstrated that the anchorage-dependent and independent proliferation of cancer cells was inhibited in c-Jun transactivation mutant which confirmed the critical role of the c-Jun/AP-1 complex. The studies also unveiled a novel mechanism by which the protein kinase Akt regulates the c-Jun activity in pancreatic cancer cells (Shin et al., 2009). Their studies revealed that the Akt regulated the transcriptional activity of c-Jun, independent of the phosphorylation
sites usually targeted by the c-Jun NH₂-terminal kinase (Ser⁶³/Ser⁷³) and glycogen synthase kinase-3 (Thr²³⁹).

The acute or chronic loss of hepatic function caused by alcohol, viral infection, or other hepatotoxic drugs can result in severe illness such as fulminant hepatitis, or cirrhosis, and greatly increases the risk for the eventual development of hepatocellular carcinoma (Okuda, 2000). Apart from this, chronic infections with the hepatitis B virus (HBV) and the hepatitis C virus (HCV) represent major risk factors for hepatocellular carcinoma (Okuda, 2000). Interestingly, AP-1 was reported to be activated in both the hepatocellular carcinoma and chronic hepatitis (Liu et al., 2002). In vitro studies using liver-derived cell lines have demonstrated rapid activation of AP-1 by HBV or HCV proteins (Kato et al., 2000). Thus, there are ample evidences demonstrating the role of c-Jun or other AP-1 proteins in liver cancer.

Role of c-Jun in apoptosis

Apoptosis is a phenomenon that eliminates undesired cells during development, immune responses and oncogenic transformation. The c-Jun is known to function as both a positive and a negative regulator of apoptosis (Shaulian and Karin, 2002). The c-Jun and JNK both have been implicated in the induction of programmed cell death (Xia et al., 1998). It is quite interesting to note the marked ability of a single transcription factor, c-Jun to compliment the alternate pathways of growth, proliferation and apoptosis. The varied functions of c-Jun are likely to be cell type specific and stimulus specific. Thus, the most critical question at present is; as to how can c-Jun prevent/promote apoptosis? The ability of c-Jun to function as an anti-apoptotic protein became evident from the experiments conducted by Eferl et al., (2003b); involving c-Jun-deficient hepatocytes which were found to be more sensitive to TNFα-induced apoptosis in comparison to the hepatocytes expressing c-Jun. This hypersensitivity was possibly rescued by a p53 deficiency in c-Jun deficient hepatocytes (Eferl et al., 2003b). Thus, this interesting antagonism between c-Jun and p53, a major tumor suppressor, demonstrate the ability of c-Jun to function as a tumor-promoter in the p53 deficient mice. These results together demonstrate a fine balance and controlled
regulation between the c-Jun and p53 activities. However, the exact mechanisms of how the c-Jun and p53 execute their antagonistic effects remain largely unknown. The c-Jun deficient fibroblasts exhibit elevated expression of p53 and its target gene p21waf1, which encodes an inhibitor of cyclin-dependent kinases (Schreiber et al., 1999). The elevated levels of p53 and p21waf1 were indeed responsible for the proliferation defect observed in the c-Jun deficient cells. Elevated p53 and p21waf1 expression is also responsible for the extended UV-induced growth arrest in c-jun−/− fibroblasts (Shaulian et al., 2000). Extensive research by Eferl et al., (2003b) showed that c-Jun-deficient liver tumors accumulate high levels of p53 protein, just like c-Jun-deficient fibroblasts. Interestingly, as observed in c-Jun deficient fibroblasts, the elevated levels of p53 could not be detected in c-Jun-deficient hepatocytes also. This can be explained by a rather simple argument. When normal liver cells are exposed to a tumor initiator (carcinogen), both c-Jun and p53 are induced and c-Jun can antagonize the pro-apoptotic/anti-proliferative activities of p53 (initiation stage) and the cells undergo rapid proliferation. Therefore, c-Jun-deficient cells would be eliminated more readily from the proliferating cell population than the wild-type cells through p53-mediated apoptosis. This accounts for the reduced tumor incidence in the cells devoid of c-Jun, pointing to its involvement and vital role in carcinogenesis. However, once the cells are transformed, they become insensitive to the anti-proliferative activity of p53 (representing an early cancer stage). However, in the more advanced stages of cancer, most of the tumor cells (transformed) have incurred p53 gene mutations rendering p53 non-functional. This results in an uncontrolled proliferation of cells even in the absence of c-Jun pointing to its role in tumor progression at early stages of cancer. Based on this knowledge, c-Jun inhibitors may be particularly useful during pre-cancerous states like cirrhosis or chronic viral infection, as chemopreventive agents, but rather useless in the therapy of advanced p53-mutated tumors. Based on this existing literature, the designing of the stage specific c-Jun inhibitors require a careful analysis and consideration. The availability of such inhibitors may further clarify how c-Jun acts to promote liver cancer and possibly other forms of cancer as well.
Regulation of c-jun

The c-jun/AP-1 gene product has been implicated in various biological processes which are required for normal growth, proliferation and apoptosis. The c-jun expression within a cell is primarily determined by the type of the cell and on the regulatory influences it is receiving. Thus, the spatial and temporal expression of this protooncogene is of prime importance in determining the fate of the cell. Thus, it is important to understand the complex regulatory circuit and the molecular mechanism(s) governing the modulation of c-jun gene transcription. Its regulation occurs at two levels: (i) over expression due to its enhanced transcription and (ii) due to post-translational modifications.

1. Transcriptional regulation of c-jun

For the primary analysis involving an understanding of the c-jun regulation, its genomic copy was isolated and its primary structure and transcription pattern was determined (Hattori et al., 1988). It was found that c-jun is an intron-less gene. The c-jun mRNA has one of the longest 5' untranslated stretch of about 1 kb length. The basis for the transcriptional regulation was found in the 5' noncoding sequence of the gene. This region of c-jun has a higher G+C content and the 3' noncoding sequence was rich in A+T sequence. The sequence elements spanning the 3' region represent a characteristic of a highly unstable mRNA. Such sequence elements with a shorter half life are also present in several other genes including c-fos and IL-2 (Curran et al., 1984; Shaw and Kamen, 1986). It has been reported that Jun protein synthesis enhances only 3-4 fold despite a 15-fold increase in the c-jun mRNA levels upon induction with TPA (Angel et al., 1988b). This translational inefficiency might be an intrinsic property of the c-jun mRNA sequence or is due to the action of some regulatory protein interacting with the c-jun transcript.

The upstream regulatory sequences present in the human c-jun promoter are SP-1 binding sites, CAAT box and the AP-1 binding sites. The c-jun consists of two non-conventional TATA boxes that function as a cluster for transcription initiation. Two AP-1 binding sites, spanning positions -71 to -64 and -190 to -183...
that bind to dimers of c-Jun and ATF-2, mediate inducibility of c-Jun by phorbol esters, UV irradiation, E1A, and IL-1 (Angel et al., 1988b; Van Dam et al., 1990; Stein et al., 1992; Muegge et al., 1993; Herr et al., 1994). In addition to this, a consensus CAAT site and a GC site being recognized by SP-1 transcription factor constitute the c-jun promoter. Another transcription factor binding site which mediates c-jun trans-activation in response to TNFα and phorbol esters in proliferating HL60 (myeloid leukemia) cells was identified by Brach et al., (1992). This site spans -141 to -131 region of c-jun and binds to NF-jun (characteristics similar to NF-kB) localized in cytoplasm of the untreated cells. However, upon treatment with extracellular response agents, it rapidly translocated to the nucleus.

The variant AP-1 sites of the c-jun promoter seem to be the key components responsible for the induction response. The presence of these sites upstream of the c-jun promoter suggested that its transcription can be mediated by the positive autoregulatory loop. The consensus binding site of GCN4 is closely related to that of the transcription factor AP-1 and similarities between Jun and GCN4 stimulated an investigation of the relationship between the Jun and AP-1. Transcription factor AP-1 (activator protein 1) was described initially as a DNA binding protein from HeLa cell extracts that specifically recognize the enhancer elements of SV40 and the human metallothionein IIA (hMIIA) gene. AP-1 binding sites also occur in the control regions of viral and cellular genes that are stimulated by treatment of cells with phorbol esters. Indeed, tandemly linked AP-1 binding sites mediate the elevated transcription of heterologous genes after phorbol ester treatment. Furthermore, sequencing of AP-1 tryptic peptides revealed that several fragments were identical to portions of the predicted amino acid sequence of c-Jun. These data, together with the finding that a Jun polypeptide expressed in E. coli binds to the AP-1 site, provided compelling evidence to the fact that c-Jun is a component of AP-1. However, AP-1 preparations contain several proteins and many of the AP-1 peptides are unrelated to Jun. Indeed, some of the AP-1 peptides have now been identified in the predicted amino acid sequence of JunB. Several other proteins that are covered by an umbrella term AP-1, have subsequently been identified. The fos oncogene is one of them which is responsible for the induction of osteogenic carcinomas by the FBJ murine
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sarcoma virus. Thus, the ability of JunB and JunD to bind to AP-1 by themselves alone or in conjunction with the different Fos proteins, suggested a crucial role of these proteins in c-jun transcriptional regulation. Regardless of the exact role of these different Jun proteins for basal or induced expression of c-Jun in He La cells, mutations in the AP-1 sites in the c-jun promoter abolished its response to TPA and c-Jun homodimers. This became the first direct evidence demonstrating as to how an eukaryotic cellular gene transcription can be regulated by its own gene product. Thus, the positive auto-regulation of c-jun is responsible for signal amplification and conversion of transient signals generated at the cell surface receptors into a long lasting transcriptional response. The interaction of these regulatory sites with their respective trans-acting proteins results in the positive regulation of c-jun gene. Most important feature of this control system is that the expression of c-jun gene is positively autoregulated by Jun/AP-1 itself (Angel et al., 1988). Similar to the transcriptional regulation of c-Jun expression, junB expression also occurs in a coordinated fashion, while junD expression is relatively insensitive to the agents that control proliferation and differentiation.

Depending on the cell type and conditions, other elements seem to exert prominent influence on the c-jun promoter activity. For example, in U937 cells transfected with short c-jun promoter construct (-132 to +170), phorbol ester inducibility is only partially observed when the proximal AP-1 site was not present (Unlap et al., 1992). Although, destruction of the CTF-binding site has no effect, mutation of CTF and AP-1 binding site, abolishes phorbol ester inducibility. In contrast, mutation in the SP-1 binding site enhances induction by phorbol esters. However, with longer promoter constructs phorbol ester inducibility was independent of the proximal sites, suggesting that the upstream sites can take over and behave dominantly. Thus, c-Jun induction in different cell types by different cellular responses suggested that transcription factors participate in diverse genetic programs. An example of this comes from TGF-β signalling, wherein it induces c-Jun and JunB synthesis in human lung adenocarcinoma 549 cells, AKR-2B mouse embryo fibroblasts and in K562 human erythroleukemia cells. While the growth of 549 cells is inhibited by TGF-β, the growth of AKR-2B is stimulated and growth of K562 cells was not affected at all (Pertovaara et al.,
Thus, whether an agent induces or represses the transcription of a gene solely depends on the differentiation state of the cell. Estrogens induce \textit{c-jun} transcription in all uterine cell types, while \textit{junB} is induced only in uterine epithelial cells (webb \textit{et al.}, 1993). Contrary to this, anti-estrogen, tamoxifen induces only \textit{junB} transcription in rat uterus, while not influencing \textit{c-jun} gene expression at all (Nephew \textit{et al.}, 1993).

Transcriptional regulation of \textit{c-jun} is also known to be inhibited by agents that elevate the cellular cAMP levels, and is discontinued shortly after initiation by inducing agents (Angel \textit{et al.}, 1988b; Mechta \textit{et al.}, 1989). However, neither the promoter sequences nor the factors responsible for this negative regulation have been identified so far.

2. Post translational modification of c-Jun

The activity of a transcription factors can be regulated mainly by two ways: regulation of binding of the protein to its promoter element and, when bound, regulation of its trans-activating potential. As expected from the basic partition of the protein into trans-activation domain and DNA-binding dimerization domains, the trans-activating function of \textit{c-jun} is regulated by modification of its trans-activation domain, while DNA-binding is modulated by modification of the DNA-binding domain. Homo-or heterodimerization of Jun with other leucine zipper containing proteins is a prerequisite for DNA binding and is subject to modification by the fact that the c-Jun can also associate with non-leucine zipper proteins.

Inhibition of c-Jun's DNA binding activity by phosphorylation and oxidation of SH-groups

Two dimensional tryptic digests of c-Jun, immunoprecipitated from untreated and phorbol ester treated cells revealed that one of the phosphorylated peptides (amino acids 227-253 of human c-Jun) is dephosphorylated in treated cells. The corresponding DNA sequence (one resistant to phosphorylation) is just located upstream of the basic DNA-binding domain of the c-Jun. In non treated cells, the most heavily phosphorylated forms of c-Jun bear three phosphates at Serine 243 and 249 and at either threonine 231 or 239. Less phosphorylated
forms lack phosphates at either one of this residue or at several of them. Heavy phosphorylation is correlated with low DNA-binding ability of c-Jun and hence low trans-activation potential. c-Jun protein produced in bacteria although was able to bind to the AP-1 binding site. However, the in vitro phosphorylated using the purified glycogen synthase kinase 3 (phosphorylates specifically threonine 239, serine 243 and 249) was able to bind even more efficiently to its cognate recognition sequence. Moreover, c-Jun protein with a phenylalanine or alanine mutation which interferes with phosphorylation at all the other sites, binds better to DNA and is a much stronger trans-activator in comparison to the wild type protein. The same holds true when the ser 226 from chicken c-Jun (which corresponds to serine 243 of human c-Jun) is replaced by a non-phosphorylatable amino acid. On the contrary, the oncogenic v-Jun protein, possesses phenylalanine in the place of serine (ser 216), one of the only two amino acid changes in the carboxy terminal domain which distinguishes the cellular from the viral counterpart, and explains why the viral protein is a stronger trans-activator than its cellular homologue. In addition to phorbol esters, several other agents such as UV radiations and several transforming oncogenes, reduce the phosphorylation status of the DNA-binding domain. The intracellular levels of available Jun-binding sites also influence the phosphorylation state of this domain.

The intriguing question arises as to how an inducing stimulus decreases the phosphorylation state of c-Jun’s DNA-binding domain and can increase its DNA-binding capacity? The answer to this lies in that they may either activate a specific phosphatase or inhibit a protein kinase to bring about a significant decrease in protein phosphorylation status. A completely new concept based on induced dephosphorylation of c-Jun has been proposed (Black et al., 1994). Several phosphorylation sites in the amino terminal part of the c-Jun (serines at 63 and 73, threonines at 91 and 93) determine the phosphorylation state of the DNA-binding domain. The phosphorylation of the amino terminal part alters the conformation of the DNA binding domain, thus changing the accessibility of this domain for kinases and phosphatases. This suggests that the enzymes working on the C-terminal substrates need not be regulated independently.
Cysteine residues of several transcription factors, though not directly involved in mediating DNA binding, modulate DNA binding activity in response to the subtle fluctuations in the cellular redox state (McBride et al., 1992; Pognonec et al., 1992; Guehmann et al., 1992; Arnone et al., 1995; Nakshatri et al., 1996). The studies have clearly shown that like other transcription factors, c-Jun's DNA binding and hence its trans-activating capacity, is also subject to the redox regulation, apart from the well-studied regulation by phosphorylation (Abate et al., 1990). This is shown by treating bacterially expressed c-Jun, encompassing the carboxy-terminal part of the protein (224-334 amino acid) with either N-ethyl maleimide (NEM) or the sulfhydryl oxidizing agent diazenedicarboxylic acid. Both treatments inhibit the binding of c-Jun homodimers or c-Jun/Fos heterodimers to the TPA response element (TRE) (Datta et al., 1992; Devary et al., 1992). On the other hand, addition of the large amounts of the reducing agent such as DTT enhanced the DNA binding activity of the AP-1 (Nikitovic et al., 1998). These results suggest that a cysteine is the target of redox regulation. Based on these results, further investigations revealed the role of the only two conserved cysteine residues at the carboxyl terminal of c-Jun. Mutation of the cysteine residue in the basic region of c-Jun (cys 269) enhances its DNA binding activity, at least when only the DNA binding/dimerization domain of c-Jun is used in the binding assay. This binding activity was not influenced under the reducing or oxidizing conditions. In contrast, Jun peptides in which the second cysteine was mutated were still subjected to regulation by oxidation (Abate et al., 1990).

Studies from our laboratory have also demonstrated that 2-mercaptobenzylglycine (2-MPG) modulates the expression of several protooncogenes including c-jun, H-ras, c-myc and c-fos. This potent hepatotrophic drug alters the gene expression profiles by affecting the levels of PKC. It has been speculated in the study that since PKC has tandem repeats of cysteine-rich sequences, 2-MPG being the most potent reducing agent of all the -SH compounds might destabilize the PKC and in turn resist the overexpression of these oncogenes (Sharma et al., 1995; Ohri et al., 2002).
Recent studies have also shown a role of reactive oxygen species (ROS) as an important mediator of proliferation, which acts as a second messenger to modulate the activation of several signalling molecules and pathways. Although, high levels of ROS may induce modifications that inhibit the activity of cellular components or result in damage, repair and cell death, low levels of ROS are crucial for signalling pathways and regulating essential cellular functions (Torres, 2003).

**Negative regulation of c-jun**

The negative regulation of c-jun is essential for normal functioning of cells as c-jun is at a risk of being permanently activated or overexpressed due to its characteristic feature of positive autoregulation. Since, the overexpression of c-Jun is lethal and leads to neoplastic transformations, cells must employ a negative regulatory circuit to curtail the overexpression of c-jun gene. Role of the upstream regulatory sequences have given an insight into the cis-elements involved in the negative regulation of c-jun. It has been shown that the deletion of the SP-1 and CTF sites of the c-jun promoter results in enhancement in both its basal and induced activity by approximately 10 folds (Angel, Hattori et al., 1988). However, it is yet to be determined if both the sites work together to negatively regulate c-jun promoter activity or one of these is sufficient by itself. Also, which of the two sites is responsible for decreasing c-jun promoter activity and how this negative regulation is actually exerted, is not known. Another target for negative regulation is the AP-1 site of c-jun promoter. This site is recognized by homodimers and heterodimers of JunB and junD. While c-Jun is an efficient activator of c-jun promoter, experiments have indicated that JunB and JunD are not (Chiu et al., 1989). JunB acts as a negative regulator and a 4 fold excess of JunB over c-Jun inhibits trans-activation of AP-1 response genes by c-Jun (Schutte et al., 1989; Chiu et al., 1989). However, the mechanism underlying repression conferred by JunB is not clear. Also, in contrast to the c-Jun homodimers, heterodimers formed by JunB and c-Jun lower the transcription efficiency of c-jun. Interestingly, JunB acts as a negative regulator on promoters with a sole AP-1 site, but it is as effective as c-Jun as an activator on promoters with multiple AP-1 sites. JunD is
also characterized as an inefficient activator of c-jun gene transcription like JunB although both have potential to bind to the AP-1 consensus site. Therefore, their constitutive expression doesn’t enhance c-jun transcription. However, it has been demonstrated experimentally that both these Jun proteins are inactive as trans-activators only in the absence of c-Fos protein (Hirai et al., 1989). Thus, it can be speculated that the constitutive expression of JunB and JunD maintains the cellular homeostasis of c-Jun, keeping its expression at the basal level until the cell is appropriately stimulated.

Search for several proteins possessing Jun inhibitory activity, revealed a labile protein, IP-1, which can interact with the leucine zippers of Jun and Fos. IP-1 is inactivated upon phosphorylation by a cAMP-dependent protein kinase A and is reactivated following dephosphorylation (Auwerx and Sassone-Corsi, 1991). Jif-1 is another protein which inhibits the DNA binding and trans-activating ability of Jun in vitro by interacting with the leucine zipper of c-Jun (Monteclaro and Vogt, 1993)

Coactivators of Jun

Extensive studies have shown that the trans-activation domain of c-Jun interacts directly with the TATA binding protein associated factors (TAFs) and activate the RNA polymerase II mediated preinitiation complex formation, and do not require coactivators per se to initiate the transcription. On the contrary, a coactivator of the CREB-binding protein (CBP, a 300-kDa protein) binds to the activated CREB when it is phosphorylated at Ser 133 but not to inactive, nonphosphorylated CREB and stimulates transcriptional activation through CREB (Kwok et al., 1994). Studies employing the CBP overexpression or inactivation suggest that the CBP is a coactivator not only for CREB, but also modulates the expression of c-Jun and c-Fos (Arias et al., 1994; Kouzarides, 1995a, b; Bannister and Kouzarides, 1995). In vitro and in vivo studies suggest that CBP binds to the amino-terminal domain of the c-Jun and the presence of Ser 63 and 73 in c-Jun is essential for this interaction. However, whether these serine residues need to be phosphorylated or not is not clear (Arias, et al., 1994, Bannister et al., 1995). Further studies on similar lines by Claret et al., (1996), revealed a protein, JABI, which interacts with c-Jun and Jun D, but not with Jun B or v-Jun. Thus, JABI has
the potential to selectively trans-activate either the c-Jun or Jun D. The N-terminal of the JABI was found to be similar to the N-terminal region of Pad1, identified as a co-activator of a subset of AP-1 target genes from the yeast (Shimanukai et al., 1995). JABI was able to efficiently trans-activate c-Jun with an efficiency almost equivalent to that observed with the previously identified coactivator, CBP in transient transfection experiments involving overexpression of both the proteins (Arias et al., 1994). Since the JABI and Pad1 are functionally interchangeable based on their sequence similarities, they form a new group of coactivators that increase the specificity of target gene activation by AP-1 proteins. Although, the exact biological function of JABI in mammalian cells has not been elucidated, the remarkable functional conservation between JABI and Pad1, especially the ability of JABI to confer drug resistance, suggests that it might regulate drug-resistance genes. In this respect, it is interesting to note that the known external stimuli such as Ras, alkylating agents or ultraviolet irradiation (Chin et al., 1992a, b), on the c-jun and AP-1 activity (Hibi et al., 1993; Van Dam et al., 2001) also stimulate the expression of the human multidrug-resistance genes.

**Role of transcription factors in regulating gene expression**

Transcription factors or the DNA binding proteins regulate the activity of the adjacent genes by interacting with either the enhancer or promoter regions present upstream of the respective genes. Depending on the activity of the transcription factor, the expression of the gene is either up- or down-regulated. Transcription factors use a variety of mechanism(s) for the regulation of gene expression (Gill, 2001). These mechanisms include either, the stabilization or blocking the interaction of the RNA polymerase at the core promoter or by catalyzing the acylation or deacylation of the chromatin. To achieve this, the transcription factors recruit accessory proteins possessing the catalytic function at the site of action. Usually, one of the two mechanisms is employed by majority of the transcription factors to regulate the gene transcription (Narlikar et al., 2002). The two major proteins which are involved in the overall process are histone acetyltransferase (HAT) and histone deacetylase (HDAC). HAT activity weakens the association of the DNA with histones by acetylating the DNA thereby rendering DNA more
accessible to the transcription. On the other hand, HDAC activity strengthens the chromatin-histone association by deacetylating the DNA, thereby down regulating the transcription of the adjacent gene. The third mechanism responsible for modulating the gene transcription also exist, which is slightly complicated than the other two and requires recruitment of coactivators or corepressors at the site of the transcription (Xu et al., 1999). Transcription factors are thus conventionally termed as the group of proteins that interpret the genetic "blueprint" in the DNA by reading the overall sequence. They bind DNA and help initiate a program which either results in an increase or decrease in the gene transcription depending on the type of the mechanism followed. Some of the important functions and biological roles of transcription factors are discussed here:

**Basal transcription**

The general transcription factors (GTFs) constitute an important class of transcription factors which are prevalent in eukaryotes and as the term describes, are required for the general/basal transcription (Weinzierl, 1999; Reese, 2003; Shilatifard et al., 2003). These GTFs do not actually bind to DNA but form a part of the large transcription pre-initiation complex that interacts with RNA polymerase. This pre-initiation complex in turn binds to the promoter regions upstream of the gene which they regulate. These transcription factors do not modulate the gene expression by either overexpression or downregulation rather function to maintain the constant levels of the expressed gene products and particularly function for the expression of the housekeeping genes.

**Spatial and temporal expression and recognition of transcription factors**

Such transcription factors regulate the transcription of the genes which are required to be expressed only at a certain time and at a certain location, i.e. temporal and spatial expression of genes, and in the right amount depending on the fluctuations in the environment and requirement of the organism.

**Transcription factors involved in development Regulation**

Transcription factors in multicellular organisms are also involved in development (Lobe, 1992). Responding to cues (stimuli), these transcription
factors turn on/off the transcription of the related genes. This result in changes in cell morphology or activities needed either for the cell fate determination or cellular differentiation. The Hox protein, for example, is an important transcription factor for appropriate body pattern formation in fruit flies as well as in humans (Lemons and Ginnis, 2006; Moens and Selleri, 2006). Another well studied transcription factor encoded by the Sex-determining Region Y (SRY) gene, plays a major role in determining gender in humans (Ottolenghi et al., 2007).

**Response to the intercellular signals**

Cells can communicate with each other by releasing molecules that produce signaling cascades for the nearby receptive cell. Depending on the signal transmission within the recipient cell, the upregulation or downregulation of genes occurs, and in both the cases the transcription factors function as part of the downstream signaling cascade (Pawson, 1993). Estrogen signaling is an example of a fairly short signaling cascade that involves the estrogen receptor transcription factor. Estrogen when secreted by ovaries and placenta, crosses the cell membrane of the recipient cell and complexes with the estrogen receptor in the cell's cytoplasm. The estrogen receptor then migrates to the cell's nucleus which is followed by binding to its cognate recognition sequence, thus altering the transcription of the estrogen-dependent genes.

**Response to the environment**

Transcription factors can also respond to environmental stimuli. Few examples of such transcription factors include: the heat shock factor (HSF) upregulates genes necessary for survival at higher temperatures (Shamovsky and Nudler, 2008); hypoxia inducible factor (HIF) upregulates genes necessary for cell survival in low oxygen environments (Benizri et al., 2008) and sterol regulatory element binding protein (SREBP) helps in maintaining the appropriate levels of lipid within the cell (Weber et al., 2004).

**Transcription factors as cell cycle regulators**

Many transcription factors, especially some that are proto-oncogenes or tumor suppressors, help regulate the cell cycle and as such determine the fate of
the cell prior to the cell division (Wheaton et al., 1996; Meyyappan et al., 1996). One example is the myc, which has important roles in cell growth and apoptosis (Evan et al., 1994).

After having determined the involvement of transcription factors in diverse biological processes, it is important to understand the regulation and control of several biological processes mediated by these diverse groups of transcription factors. The role of transcription factors is to not only control the rate of transcription of associated genes but also to regulate the amount of gene products available to the cell. Thus, a more stringent and precise regulation for the expression of transcription factors is expected. The activity of transcription factors can be regulated at several levels such as synthesis, translocation, activation etc. which are briefly described below:

At the level of synthesis

Since the transcription factors are also proteins and are transcribed from a gene and translated into a protein, their production can be regulated at any of these two steps. The transcription factors possess mechanisms by which they can positively or negatively auto-regulate (feed-back mechanism) their own expression by functioning either as enhancers or repressors, respectively. Both these mechanism(s) help maintaining the appropriate levels of transcription factors within the cell.

Nuclear translocation

In eukaryotes, all the proteins are transcribed in the nucleus and are then translated in the cell's cytoplasm. Many proteins that are active in the nucleus contain nuclear localization signals (NLS) that direct them to the nucleus. For many transcription factors, the key point in their regulation is their binding to a ligand while in the cytoplasm before they can relocate to the nucleus. The absence of any signal sequence in some of the transcription factors points to an alternate mechanism of their export within the nucleus. It has been speculated that some transcription factors have the general ability to interact with the nuclear resident proteins which might function as cargo machines in the cellular cytoplasm for
mediating their nuclear import. Such small protein molecules include importins, Ran etc. and have the ability to cross the nuclear membrane with ease (Whiteside and Goodbourn, 1993).

**Activation in response to modification of transcription factors**

Transcription factors may be activated (or deactivated) through their transactivation domain by a number of mechanisms including ligand binding, ability of transcription factors to bind to other cofactors or other transcription factors (e.g., homo- or hetero-dimerization) or co-regulatory proteins and phosphorylation. The best studied example to this category of regulation is the activation of NF-kB dimers, which are sequestered in the cytoplasm as an inactive complex by the group of inhibitory molecules, IκB. IκB’ are known to mask the NLS sequence of NFκB hampering its translocation to the nucleus. Following phosphorylation of IκB, the inhibitors tend to dissociate (because of their ubiquitin mediated degradation) promoting the translocation of NF-kB to the nucleus allowing it to execute its effects.

**Availability of other cofactors/transcription factors**

Generally, transcription factors do not function in isolation and work in concert with other factors. Often, for gene transcription to occur, a number of transcription factors must bind to the cognate recognition sequence. The combination of several transcription factors in turn recruits intermediary proteins such as cofactors that allow efficient recruitment of the pre-initiation complex and the RNA polymerase. Thus, for a single transcription factor to initiate the transcription, all the coactivators and regulatory proteins must be present for efficient activity of the transcription factors.

**GFP as a choice of reporter gene**

The cloning of the wild type GFP gene of the jelly fish *Aequorea Victoria* and its subsequent expression in heterologous systems has established GFP as a powerful reporter for the analysis of gene expression and protein localization in a wide variety of experimental systems. When the gene is expressed in either eukaryotic or prokaryotic cells and illuminated by excitatory light, GFP emits a
bright fluorescence that is easily detected by light. The GFP chromophore consists of a cyclic tripeptide derived from Ser-Tyr-Gly at positions 65-67 in the protein and is only fluorescent when embedded within the fully folded, complete GFP molecule. This provides the proper environment for the chromophore to fluoresce by excluding solvent and oxygen. However, nascent GFP is not fluorescent, since the chromophore formation occurs post-translationally. The chromophore is formed by a cyclization reaction and an oxidation step at Tyr66 that requires molecular oxygen. Therefore, this chromophore formation may be rate limiting especially if oxygen is limiting. Light stimulated GFP fluorescence is species-independent and does not require additional cofactors, substrates or gene products from the source. In addition to its expression alone, GFP has also been used extensively to express GFP fusions with a variety of other proteins and targeting sequences. The chimeric genes encoding either the N-or C-terminal fusions to GFP retain the normal biological activity of the heterologous partner, as well as maintain the full fluorescent properties of the native GFP.

Figure 3: (A) Transcription and translation of a reporter gene. The amount of protein expressed will depend on the strength of the promoter. (B) The $\beta$-can structure of the green fluorescent protein (GFP) (PDB 1GFL) of *Aequorea victoria*
The use of GFP and its variants in this capacity provides a "fluorescent tag" on the protein, which allows an in vivo localization of the fusion protein. GFP fusions provide enhanced sensitivity and resolution compared to standard antibody staining techniques. GFP tag eliminates cell fixation, permeabilization and antibody incubation steps that are required in standard immunofluorescence staining methods. The expression of GFP also offers the opportunity to conveniently monitor gene expression at single cell level and can be evaluated for the purpose of monitoring both the magnitude and duration of gene expression in transient expression systems.

Use of GFP as a transcription reporter is due to its compact β-can structure which renders this protein extremely stable and resistant to degradation by most cellular proteases. In fact, the half life of EGFP expressed in the cytoplasm of mammalian cells has been estimated to be greater than 24 h. This level of stability is an advantage for many applications for which a stable fluorescence signal from GFP is desirable, but a serious disadvantage in applications to monitor changes in gene expression. In transcription reporter assays, changes in the level of the reporter protein are presumed to reflect changes in mRNA levels resulting from either induction or repression of cis-acting control elements linked to the reporter gene (Figure 3).