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The Structure and Function of Eukaryotic Enhancer Elements and their Role in Oncogenesis

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Abstract. *Transcriptional enhancers have now been identified near or within a large number of viral and cellular genes. They are cis-acting regulatory sequences generally capable of elevating transcription from a linked gene in a distance and orientation independent manner. More recent evidence suggests that this activity may be modulated in a host-cell-specific manner. Enhancer function in some cases at least appears to be controlled by the operation of trans-acting factors within the host cell. Occasionally enhancers may also be responsible for repression of gene transcription. It has now become clear that the development of some cancers may be linked with the activity of enhancer sequences. It is likely that this process involves subversion of the normal regulatory mechanisms which control expression of proto-oncogenes through the interposition of exogenous, cis-acting enhancer sequences. The evidence comes from two sources, firstly a strong association between the action of enhancer sequences and some forms of naturally occurring cancers, and secondly, the induction of neoplastic transformation in susceptible cells by genetically manipulated oncogenes under the transcriptional control of enhancer elements.*

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1. Introduction

Expression of eukaryotic protein coding genes depends on a multi-step series of regulatory mechanisms. The most frequent level of regulation appears however to be control at the level of mRNA synthesis (1), and is dependent on the interaction of RNA polymerase II with specific DNA signal sequences, or promoter elements, located in the flanking regions surrounding the gene or occasionally within the gene itself. Over the past few years it has become increasingly clear that the organization of such signal sequences is not in a general sense rigidly controlled and depends very much on the individual gene concerned. However,some general rules do apply. All genes possess a TATA consensus sequence or functional equivalent which is responsible for localization of the mRNA start point. All genes in addition possess upstream promoter elements of some sort which are responsible for the control of efficient gene transcription. The upstream promoter elements of a large number of viral and some cellular genes appear to contain an additional type of regulatory element responsible for further elevated levels of gene transcription. This regulatory element has been suitably named an 'enhancer' (2). In this paper we review recent research on the structure and function of eukaryotic enhancer elements, and describe experiments which implicate enhancers in the process of oncogenesis.

2. Functional Organization of Enhancers

Enhancer sequences were first discovered within the genome of papovaviruses simian virus 40 (SV40) (2-5) and polyoma (6). Concurrent studies on the activation of v-onc (7,8) and c-onc (9) sequences by the transcriptional control signals within retroviral long terminal repeats (LTR) also demons-

trated the existence of enhancer sequences within the retroviral LTR. Nevertheless, the full implications of the enhancer effect were not realised until Barnerji *et al* (2) and in retrospect Capecchi (10) added the significant observation that for SV40 at least the enhancer sequences could exert their effect not only on expression of their natural gene, but also on the expression of heterologous genes. However, Capecchi initially proposed that the increased efficiency of transformation observed in the presence of the SV40 enhancer element was due to increased integration frequencies. Some enhancer sequences have in fact been identified solely by their ability to enhance expression of foreign genes in a non-homologous environment (11) or by sequence homology with known enhancer elements (12, 87) and therefore in some cases, less is known about their endogenous function.

Within the past few years, detailed studies have been carried out on a number of viral enhancer sequences, most notably those of SV40 (4, 13, 14) but also polyoma (6, 15), Moloney sarcoma virus (MSV) (16, 17) and the herpes simplex virus type 1 IE mRNA3 gene (18, 19). These studies have allowed a working definition of an enhancer element to be established, and have uncovered a number of important properties attributable to enhancer elements. These properties are listed in Table I. Firstly, an enhancer element with endogenous promoter attached is capable of elevating the inherent level of transcription from a foreign linked gene whose own promoter has been removed. This has been demonstrated for the effect of the SV40 enhancer on E. coli gpt (20), Tn9 chloramphenicol acetyltransferase (21), E. coli galactokinase (22) and herpes simplex virus thymidine kinase (tk) (17), also for the Rous sarcoma virus LTR promoter on Tn9 chloramphenicol acetyltransferase (21), the avian leukosis virus (ALV) LTR promoter on c-*myc* (23, 24), the Harvey murine sarcoma virus (HaMuSV) LTR promoter on v-*ras* (8) and the Moloney murine sarcoma virus (MoMuSV) LTR promoter on v-*mos* (7) and herpes virus *tk* (17). In addition, the herpes simplex virus type I immediate early mRNA 3 promoter can elevate expression of herpes virus tk (18). Secondly, an enhancer element with or without an endogenous promoter may be linked to a gene whose promoter remains intact. In this case, an enhancer is capable of elevating the basal level of transcription directed from the natural gene promoter. This property has been described for the effect of the enhancers of SV40 on large T antigen under control of the chicken conalbumin or adenovirus 2 major late promoter (13, 14) and on herpes virus tk (10, 17), rabbit ß-globin (2), chicken lysozyme (25), mouse immunoglobulin light chain (26) and β^0 thalassaemic human globin (27), of bovine papilloma virus (BPV) (28, 29), adenovirus (30), MoMuSV (16, 17), and a human DNA-SV40 homologue (12) on herpes virus tk, and of polyoma on rabbit ß-globin (31) and herpes virus tk (32). Thirdly, an enhancer element alone linked to a promoterless gene appears to be capable of directing initiation of gene transcription from downstream cryptic start sites, albeit at a substantially lower level than in the presence of a functional promoter element. This effect has been described for the enhancers of SV40 (13, 14), BPV (28) and MoMuSV (17).

Table I. *Properties of enhancer elements.*

1. Cis-acting
2. Positive regulatory elements[a]
3. Function independent of orientation
4. Effective over relatively long distance
5. Demonstrate a degree of host cell specificity
6. Regulated by trans-acting factors[b]
7. Possess a consensus sequence

a: Some enhancer sequences are also capable of negative regulation (see Section 2).

b: While the property of trans-activation has been documented for only a few enhancer sequences, it is conceivable that most, if not all enhancer sequences may be targets for interaction with specific DNA binding proteins.

Interpretation of the above data therefore suggests that enhancer sequences are capable of elevating the level of transcription from any eukaryotic gene with the pre-condition that a promoter element is juxtapositioned between the 5' end of the gene and the enhancer sequence. In general, the origin of the promoter sequence is unimportant and may be endogenous to the gene, endogenous to the enhancer or a foreign promoter altogether. In addition, provided a downstream promoter is present, the insertion of an enhancer does not alter the location of the start point for mRNA transcription (14). Moreover, enhancer sequences appear capable of initiating transcription in the absence of a downstream promoter element, although in this case transcription is much less efficient.

Enhancer sequences possess two other important properties. They appear to be capable of operating in either orientation and at a distance of up to a few kb from the mRNA start point. Thus, the ability to operate in either orientation has been demonstrated for the enhancers of ALV (23), BPV (28), polyoma (31), MoMuSV (16, 17), SV40 (2, 13, 14), herpes simplex virus type 1 immediate early mRNA3 (18), the sea urchin H2A histone gene (33, 34) and a human-SV40 homologue (12). However, the enhancer sequences of human cytomegalovirus function unidirectionally only (35). That enhancers can exert their effect over a distance has been observed for most enhancers so far studied but has been particularly well described for SV40 (2, 13, 14, 17). However, Wasylyk et al (36) have recently studied the distance effect of enhancer function in greater detail and have shown that, for SV40 at least, this effect can be separated into two distinct activities, one acting over a short range of around 150 bp, the other over a longer range of up to a few kb. Wang and Calame (37) have further shown that the immunoglobulin heavy chain enhancer can activate promoter sequences

located over 17 kb away, a distance much greater than previously reported for any other enhancer sequence.

Growing evidence suggests that subtle functional differences do occur between enhancer elements. They are capable of acting in a host specific manner. The degree of host specificity appears to vary between enhancers and ranges from a broad host range with a degree of host cell preference for the enhancers of SV40 (38-41) to an absolute host cell specificity apparent for immunoglobulin enhancers (42-44). In addition to SV40 and immunoglobulin enhancer sequences, host cell specificity has also been demonstrated for the enhancer sequences of BPV (41), HaMuSV (45), MoMuSV (38), MuLV (47, 48, 50), Rauscher mink cell focus-inducing virus (MCF) (51), myeloproliferative sarcoma virus (MPSV) (52) and polyoma (39). The release of an expression block, allowing polyoma virus to grow in mouse embryonal carcinoma cells correlates with sequence alterations within the enhancer and is probably due also to a host - specific interaction (49).

However, Mason *et al* (53) and Foster *et al* (54) have also demonstrated that the immunoglobulin heavy chain and the kappa light chain gene promoter sequences exhibit powerful cell-type specificity independent of the enhancer element. Grosschedl and Baltimore (55) have identified a further regulatory region with the heavy chain intragenic region which similarly confers cell-type specificity on the IgH gene. There appears therefore to be at least three separate functional domains responsible for the observed cell-type specificity of Ig gene transcription.

Nevertheless, the observed host cell specificity associated with enhancer function suggests that enhancer elements themselves are the targets of specific host cellular proteins capable of regulating enhancer activity. Consequently a native enhancer sequence should compete favourably with a foreign enhancer sequence for cellular components in homologous cells. Thus competition assays have demonstrated specific cellular trans-acting factors are required for SV40 and MSV enhancer function *in vivo* (56) and SV40 enhancer function *in vitro* (57). Moreover, in a joint *in vivo* competition assay, the SV40 or MSV enhancers compete more effectively for cellular components in homologous cell systems; CV-1 cells for SV40 and LTK$^-$ cells for MSV (56). Similarly in lymphoid cells, the immunoglobulin heavy chain and SV40 enhancer sequences compete for a common factor necessary for enhancer function. However, in fibroblasts, a functionally distinct factor is utilised by the SV40 enhancer but is unavailable to the IgH enhancer (58). *In vitro* the mouse heavy chain immunoglobulin and adenovirus 2 enhancer elements compete efficiently with SV40 for enhancer activating cellular factors, while the polyoma virus enhancer does so only weakly (57).

These data suggest that a number of cellular factors may interact with enhancer elements to initiate gene transcription. The precise binding affinity of these factors with specific domains on enhancers may reflect subtle differences between enhancers and may explain the observed cell specificity of function characteristic of most enhancer sequences.

For the most part, the cellular factors involved in enhancer interactions remain undiscovered. Hoever, Piette *et al* (59) have identified two separate factors from mouse 3T6 cells which interact with the polyoma virus enhancer. Singh *et al* (60) have likewise identified a nuclear factor (IgNF-A) which binds specifically to a sequence motif present in the mouse immunoglobulin heavy chain gene enhancer. Jones and Tjian (61) have also demonstrated the presence of Sp1 binding sites within the enhancer sequences of the HSV-1 IE mRNA3 gene. This enhancer sequence comprises a number of separate functional domains in addition to Sp1 binding sites. It is not yet clear whether the Sp1 sites exert any direct influence over IE mRNA3 enhancer function or are merely interspersed between functional enhancer elements.

It is likely that the activity of most genes controlled by enhancer sequences is regulated via specific trans-acting factors. For some genes, particularly of viral origin, the effector molecules capable of regulating enhancer function have been readily identified. For viruses detection has been especially easy since the effector molecules are usually encoded within the viral genome itself. Where such clear enhancer / effector relationships have been established, the enhancer elements have been termed "inducible".

Thus an inducible enhancer element has been identified within the promoter sequence of the HSV-1 IE mRNA3 gene (18) regulated *in trans* by a 65K viral protein (62). For BPV, an inducible enhancer has been found within the 1kb non-coding region of the genome trans-activated by the viral E2 gene product (63). The regulation of transcription from the adenovirus genome is complex and similarly involves the action of inducible enhancer elements. Early in adenovirus infection the EIA gene product is capable of stimulating transcription from its own enhancer element (64, 65). In addition E1A stimulates transcription from an inducible enhancer element controlling expression of the E2 gene (66).

The enhancer sequences located within the LTR of some retroviruses have also been shown to respond to regulation by virus encoded activator proteins. Thus Rous sarcoma virus (RSV) transcription is stimulated by a 124 residue protein originating from an alternate reading frame within the gag gene (67). Similarly human T cell leukaemia viruses HTLV I and HTLV II exhibit trans activation of the LTR by a virally encoded protein called *x-lor* or *tat* originating from the x region or long open reading frame (LOR) located between the envelope gene and the 3'LTR (46). The retrovirus associated with acquired immune deficiency syndrome (AIDS), HTLV III or LAV also possesses an inducible enhancer sequence located within the LTR between positions -17 and -137 relative to the viral cap site (68, 69). Enhancer activity here is stimulated by a trans-acting factor of 58 residues (70) encoded by a region next to the 5' end of the viral envelope gene within the second exon of a triple exon gene (70, 71). Regulation of transcription from the mouse

mammary tumour virus (MMTV) genome is similarly controlled by an inducible enhancer sequence (72, 73).In this case however, the effector molecule is glucocorticoid hormone rather than a virally encoded gene product. The inducible enhancer elements are again located within the viral LTR.

Inducible enhancers have also been identified within the eukaryotic genome. Thus expression of the human ß interferon gene is activated via an inducible enhancer element in response to viral infection or exposure to synthetic double-stranded RNA (74). In addition, the promoter domain of the

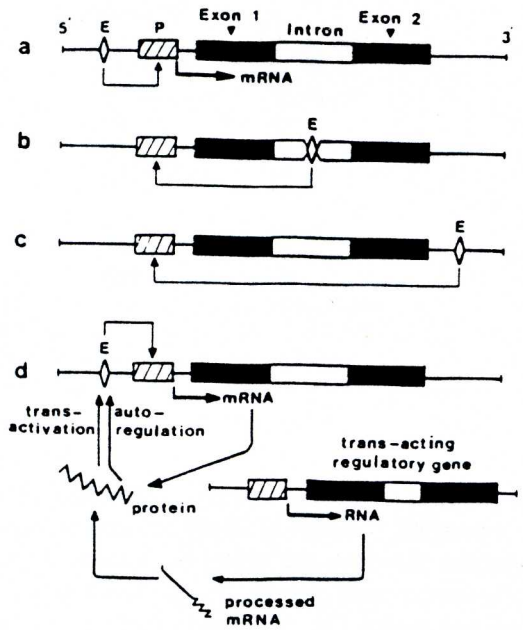

Figure 1. *A schematic representation of enhancer function. Regulation of gene transcription by enhancers may occur through cis-activation of the basic promoter sequences (hatched box) by the enhancer. The enhancer may be located upstream (a) downstream (c) or within the gene(b). The activities of at least some enhancer elements are themselves regulated by trans-acting factors which specifically interact with sequence elements within the enhancer domain (d). The trans-acting factor may interact directly with the enhancer or may do so via a further cellular component. In some cases, enhancer activity may be autoregulated (d). The above scheme of enhancer action may also be proffered for the action of enhancer sequences whose activity results instead in the direct repression of gene transcription.*

The precise mode of action of enhancers remains yet to be proven. Two major theories are currently on offer. The first, originally described by Moreau et al *(13) suggests that enhancer elements act as bi-directional «super» entry sites for RNA polymerase. Certainly, the SV40 enhancer is responsible for an increase in the number of RNA polymerase molecules found on a linked gene (175, 176). The effect over distance may be explained by condensation of DNA bound transcription factors through 'looping out' of intervening DNA (177). The second theory implicates changes in general chromatin structure or enhancer mediated attachment to the nuclear matrix as mechanisms of enhancer function (37). Further elucidation awaits the outcome of enhancer studies* in vitro.

Hatched box = promoter, shaded box = coding exons, open box = intron,

E = enhancer element, P = promoter.

ENHANCER CONSENSUS SEQUENCE

$$5'\text{-GXTGTGG}\begin{matrix}\text{TTT}\\\text{AAA}\end{matrix}\text{-}3'$$

Figure 2. *A consensus sequence for enhancers. The above consensus sequence is necessary for efficient functioning of the SV40 enhancer element (84), although little functional evidence exists for the homologous equivalents of other viral enhancers. Contrasting evidence provided by Weber* et al *(173) to some extent places doubt on the efficacy of a general consensus sequence for enhancers since spontaneously created enhancers induced by the 'enhancer trap' system possess limited homology to the described consensus. In addition, Church* et al *(174) have described a separate consensus sequence for the intron enhancers of immunoglobulin genes, and Veldman* et al *(94) further specify individual regions of the polyomavirus enhancer which respectively show homology to sequences within the enhancers of RSV, adenovirus 5 EIA, SV40 and the immunoglobulin heavy chain gene. Thus it is likely that any enhancer may comprise several types of sequence domain.*

human metallothionein II_A (hMT-IIA) gene exhibits properties resembling those of an inducible enhancer and possesses separate DNA sequence elements which respond to induction by either heavy metal ions or glucocorticoid hormones (75, 76). However, Haslinger and Karin (106) have recently shown that the enhancer sequences of hMT-IIA can direct high basal levels of gene transcription even in the absence of induction.

The above data suggests that enhancer sequences are positively regulated *in trans* by interaction with cellular factors. In contrast, more recent evidence has also shown that the activity of some enhancers at least may be repressed by trans-acting factors. Initial evidence comes from the observed repression of viral enhancer activity in undifferentiated embryonal carcinoma cells (77-79). The nature of one such repressor molecule has been elucidated, by Borelli *et al* (80) who have demonstrated repressor activity associated with the action of adenovirus 2 EIA, concurrently providing evidence that the EIA gene product may itself be autoregulated. Hen *et al* (81) have further shown EIA is capable of repressing activity of the immunoglobulin heavy chain enhancer. These experiments evoke an intriguing model of both positive and negative regulation of gene expression by a single gene, since EIA is already well known as a direct activator of gene transcription (64, 83).

It seems probable then that the activity of most if not all enhancer elements is controlled by the interaction of trans-acting effector molecules with the specific DNA sequence of the enhancer. However, trans-activation may not necessarily represent a direct interaction between the effector molecule and the enhancer element. It is conceivable that the trans-activation observed may be mediated via cellular factors after initial interaction with the primary effector molecule. Whatever the explanation, this interaction may be manifest as positive or negative regulation of transcription from the gene

Table II. *Origin of eukaryotic enhancer sequences.*

a. Viral enhancer		References	b. Cellular enhancer		References
Papovariruses:	SV40	2-5, 13, 14, 36, 90	Sea urchin H2A histone:		33,34
	Polyoma	6, 15, 31, 32, 94			
	BKV	87	Mouse major histocompatibility		123
	LPV	88	complex gene Eß:		
	JCV	89			
	BPV	28, 29, 63, 91	Mouse and human immunoglobulin:	heavy chain	42-43, 110
				kappa light chain	44, 111-116
Retroviruses:	MoMuSV	7, 16, 17		lambda light chain	117
	HaMuSV	8			
	RSV	21, 67, 97, 98	Human:	insulin	119
	ALV	23, 24		chymotrypsin	119
	MMTV	72, 73		type 1 keratin	120
	HTLVI	46		antithrombin III	121
	HTLVII	46		*fos*	163, 164
	HTLVIII	68, 69		ß-interferon	74
				metallothioncin IIA	75, 76, 106
Herpesvirus:	HSV-1	18, 19		unidentified	12
	CMV	35	Mouse:	unidentified	11
	HSV saimiri	107			
Adenovirus:		30,66,85,100			
Hepatitus B virus:		82,108			

under enhancer control. Putative models representing such control mechanisms are shown in Figure 1. Enhancers may therefore represent a convenient and important mechanism of gene control within the cell, allowing gene expression to be enhanced or repressed both during cell growth and during differentiation. A list of characterised enhancer sequences and major references are shown in Table II.

3. Structural Organization of Enhancers

There is little structural similarity between enhancers although a number of enhancers within the retrovirus and papovavirus groups demonstrate tandem duplications of a direct repeat sequence. Lack of sequence homology does not appear to reflect absolute differences in function, as enhancers seem to work efficiently within ubiquitous gene systems (13, 14, 17) and are functionally interchangeable (15, 122).

At the DNA sequence level, enhancers appear to be heterogeneous and little homology exists between species although Weiher *et al* (84) and more recently Hen *et al* (85) have identified a core consensus sequence represented within the enhancer regions of adenovirus, MSV, SV40, polyoma, BPV, and the mouse immunoglobulin heavy chain locus. The described consensus sequence for enhancers is shown in Figure 2. Whether this consensus sequence has a ubiquitous role to play in enhancer function is not yet clear. Nordheim and Rich (86) however suggest an additional mechanism of enhancer function, and postulate that Z-DNA segments within enhancer sequences modulate transcriptional activation via changes in local chromatin structure.

4. Viral Enhancers

a. Papovaviruses

While the enhancer sequences of SV40 remain the most extensively studied of all, enhancer sequences have also been identified within the genomes of other members of the papovavirus group; polyoma (6) bovine papilloma virus (28, 29, 63) and the human papovaviruses BKV (87), lymphotropic papovavirus (LPV) (88) and JC virus (JCV) (89).

With the exception of BPV, the enhancer activity is associated with a tandem repeated sequence of variable length; 72 bp for the prototype SV40 enhancer (4), 31 to 34 bp for some naturally occurring strains of polyoma virus although a 24 bp core sequence is conserved in all strains (92), 69 bp for BKV (87), 63 bp for LPV (88) and 98 bp for JCV (89).

For each virus, with the exception of BPV, the enhancer region is juxtapositioned between the starts for early and late gene transcription. Deletion studies have shown efficient expression of the early genes of both SV40 and polyoma is dependent on the presence of functional enhancer sequences (4-6). In addition, the 72 bp repeats of the canonical SV40 enhancer sequence are required for efficient induction of late gene expression (90). The enhancer sequences of polyoma have been further shown to be tightly linked with viral DNA

replication (15, 94) and to consist of multiple sequence elements (94).

In contrast to the other members of the papovavirus group, the enhancer sequences of bovine papilloma virus are located immediately 3′ to the early polyadenylation signal in the BPV genome (28, 29) and consist of two essential functional regions (minimal lenght 16 and 6 bp separated by an 8 bp non-essential region) within a 40 bp stretch of DNA (91). Presence of the enhancer region is obligatory for efficient expression of the viral early genes and for morphological transformation by the virus.

b. Retroviruses

The integrated form of an infectious retrovirus (provirus) is bounded at either end by a repeated DNA sequence called the long terminal repeat (LTR) (95,96). The LTR comprises DNA sequences originating from the 5′ end of the genomic RNA (regions R and U_5) linked to sequences originating from the 3′ end of the genome (region U_3). The promoter sequences responsible for efficient control of retroviral RNA expression appear to be located within U_3.

When enhancer sequences were first identified within the retrovirus genome, it was therefore of little surprise to find they were located within the U_3 region of the viral LTR (7-9). However, for the Prague strain of rous sarcoma virus (PrRSV) at least, an additional enhancer domain has been identified within the 3′ region of the retroviral genome in the sequences immediately preceding the LTR (97). The coordinate action of this sequence with an adjacent sequence within U_3 is necessary for full enhancer function. A similar enhancer domain has been described by Luciw *et al* (98) for the SR strain of RSV.

Like the papovaviruses, the enhancer function of retroviruses are often associated with tandem repeat sequences. However, only one copy of the repeated element appears to be necessary for enhancer function (16, 99).

The consequences of activation of powerful promoter sequences integrated within the host cell genome are far-reaching. Clearly viral enhancers within retrovirus LTRs are capable of initiating transcription from adjacent host cell genes. When such genes have oncogenic potential, their activation may initiate the process of neoplastic transformation within the host cell.

c. Other Viral Enhancers

In addition to papovaviruses, enhancer sequences have now been identified within the genomes of representatives from other major groups of the double-stranded DNA viruses; adenovirus and herpes virus. The enhancer sequences of adenovirus have been identified within the left-hand end of the viral genome and appear to be essential for cis-activation of the adenovirus EIA trancription unit. However, while the enhancer element of adenovirus 2 has been located between 155 and 178 bp from the left-hand end of the genome (85, 100) that of adenovirus 5 has been mapped between positions 195 and 353 (30). This discrepancy is surprising since the genomes of adenovirus 2 and adenovirus 5 exhibit approximately 99% sequence homology within the first 400 to 500 bp. An explanation may lie with the differing experimental systems used.

For most DNA viruses, synthesis of the earliest detectable viral transcripts appears to be mediated through an enhancer sequence. While these RNAs are transcribed from the early genes of small DNA viruses such as SV40 and polyoma, for the members of the herpes virus group, which have a much larger genome, the functional equivalents appear to be the immediate early (IE) genes (101). The herpes virus group comprises a diverse collection of large double-stranded DNA viruses. The organization of the herpes virus genome is consequently both heterogenous in nature and extremely complex. It is not surprising therefore that the functional organization of the IE transcription unit also varies between virus types. Thus, while the genome of herpes virus saimiri contains only one major IE mRNA (102), those of human cytomegalovirus (HCMV) and herpes simplex virus type 1 (HSV-1) comprise at least 4 (103, 104) and 5 (105) IE mRNAs respectively. Enhancer sequences have now been detected within the genomes of the three above members of the herpes virus group, HCMV (35), HSV-1 (18, 19) and herpes virus saimiri (107). In each case the enhancer element has been located upstream from the coding sequences of an immediate early (IE) class gene. For HSV-1 and HCMV the location of the enhancer sequences have been precisely determined, lying 331 to 110 bp upstream from the HSV-1 IE mRNA3 cap site (18) and 524 to 118 bp upstream from the cap site of the HCMV major IE gene (35). It is clear that these enhancers play a major role in regulation of the respective IE genes. The corresponding sequences of herpes virus saimiri, however, are located approximately 7 kb upstream from the sequences encoding the single IE mRNA. That this enhancer element is directly involved in regulation of IE gene transcription is yet to be shown.

In addition to the double stranded DNA viruses, an enhancer element has also been identified within the genome of the partially single stranded DNA virus human hepatitus B virus (HBV) (82, 108). This enhancer element is located upstream from the gene encoding the viral core antigen (HBcAg) and is required for efficient expression of the HBcAg gene. Surprisingly the enhancer is situated within the coding region of the putative viral reverse transcriptase gene (82).

5. Cellular Enhancers

Enhancer sequences although first discovered within the genome of viruses have now been identified within the promoter domains of a number of eukaryotic genes. Of these, the most extensively studied are the enhancer elements which control transcription of the immunoglobulin genes.

Assembly of each chain of an immunoglobulin molecule

requires rearrangement of the cellular DNA sequences which separately encode the variable (V) and constant (C) regions of the gene (109). The enhancer sequences have been located within the J-C intron of the heavy chain (42, 43, 110) kappa light chain (44, 111-116) and lambda light chain (117) immunoglobulin genes. From this position, the enhancer element is capable of activating transcription from the nearby rearranged V region promoter element and thereby allows transcription of a complete immunoglobulin gene. However, while the enhancer element is essential for initiation of immunoglobulin transcription, for the I_gH gene at least, enhancer activity appears to be dispensable once the transcription unit has been activated (118).

In addition to the immunoglobulin genes, enhancer sequences have been identified within the transcriptional control regions of a number of eukaryotic genes including the human insulin and chymotrypsin (119), type I keratin (120), antithrombin III (121) and ß-interferon (74) genes. Likewise an enhancer element has been identified within the upstream regions of the mouse major histocompatibility complex gene E_β (123).

6. The Role of Enhancer Sequences in Tumourigenesis

In recent years it has become increasingly clear that abnormal expression of cellular proto-oncogenes play a role in the genesis of cancers. It is evident that interposition of an enhancer element within or adjacent to the regulatory sequences of a proto-oncogene may alter gene expression by subverting the normal regulatory mechanisms and result in either overexpression or altered temporal expression or both. Growing evidence therefore suggests that enhancer sequences are intimately involved in the process of tumourigenesis.

The evidence comes from 2 major sources. Firstly, there is a strong association between the action of enhancer sequences and some forms of naturally occurring cancers. Secondly, the deliberate induction of neoplastic transformation in cells in tissue culture and of tumours in animals may be achieved by the introduction into susceptible cells of genetically manipulated oncogenes placed under the transcriptional control of enhancer elements.

7. Enhancers and Naturally Occurring Cancers

Evidence of enhancer induced activation of proto-oncogenes in naturally occurring cancers comes largely from an analysis of the translocation breakpoints occurring in lymphoid neoplasia of both mouse and man (124), and from abundant examples of transcriptional activation of oncogenes stimulated by the promoter sequences within the LTRs of inserted retroviral proviruses.

The genesis of B cell derived tumours of mice (murine plasmacytomas) and humans (Burkitt lymphomas) involves reciprocal translocation of the *myc* oncogene to one of the

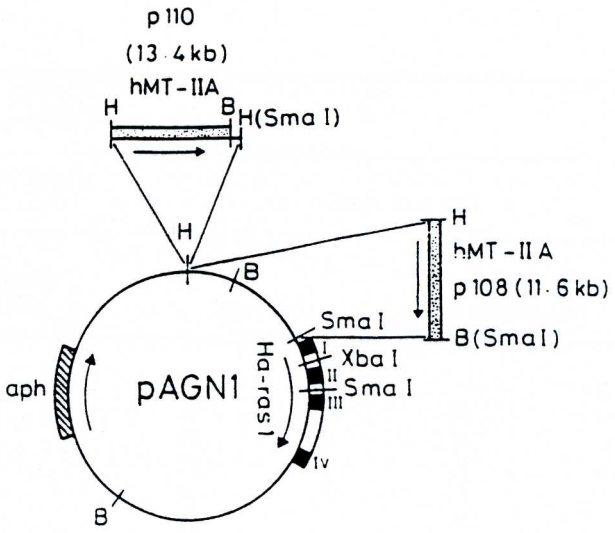

Figure 3. *A schematic representation of aph recombinant plasmids.*

Plasmid pHS1 (obtained from Dr. Michael Karin) contains the human 5' metallothionein (hMT-IIA) control sequences from +70 to −770 bp upstream of the hMT-IIA gene in the plasmid vector pUC8. Plasmid p110 was constructed by firstly converting the SmaI site of plasmid pHS1 into a HindIII site using HindIII linkers. The 0.8 kb HindIII fragment containing the hMT-IIA control sequences was then inserted into the HindIII site of pAGN1. Plasmid p108 was constructed as follows: A 602 bp SmaI fragment containing exons I and II of Ha-ras-1 was cloned into pUC8 to obtain plasmid p96 (not shown). A HindIII-BamHI fragment of plasmid pHS1 containing the hMT-IIA control sequences was then cloned 5' to the Ha-ras sequences of plasmid p96 and in the same orientation to generate plasmid p107 (not shown). The HindIII-XbaI fragment of pAGN1 carrying exon I and 5' sequences of the Ha-ras-1 gene was replaced with a HindIII-XbaI fragment of plasmid p107 containing the hMT-IIA sequences and the first exon of the Ha-ras-1 gene to obtain plasmid p108. The junction point between hMT-IIA and Ha-ras-1 in p108 is located within the untranslated leader sequence of each gene and represents a fusion between the promoter sequences of hMT-IIA 10 bp downstream from the transcription initiation site and the coding regions of Ha-ras- 1 60 bp upstream from the AUG initiation codon.

The maps are not drawn to scale; arrows represent the transcriptional orientation of Ha-ras-1, aph and hMT-IIA; B, BamHI; H, HindIII; I-IV, *coding exons of Ha-ras-1 gene.*

chromosomes carrying immunoglobulin genes. In some cases, transposition links the immunoglobulin heavy chain enhancer sequences to the coding region of c-*myc* both in Burkitt lymphomas (125-127) and in mouse plasmacytomas (128). However, in most Burkitt lymphomas and murine plasmacytomas, the immunoglobulin enhancer element is located instead on the reciprocal translocation (129-132) and is therefore unable to affect *myc* gene expression. It is likely that enhancer induced activation of c-*myc* in Burkitt lymphomas and plasmacytomas represents just one of a number of mechanisms responsible for aberrant expression of the c-*myc* oncogene. Le Beau *et al* (142) have further proposed that the recently identified enhancer sequences present within the human metallothionein IIA gene (106) may be involved in

Table III. *Transfection of rodent cells with* aph *recombinant plasmids.*

Donor DNA	No. of geneticin resistant colonies per flask									
	Rat 208F						Mouse NIH 3T3			
	Total No.			Morphologically altered (%)			Total No.		Morphologically altered (%)	
	–	+Dx	+Cd	–	+Dx	+Cd	–	+Dx	–	+Dx
pAGT1	14	12	15	86	92	73	53	62	96	94
pAGN1	12	10	13	0	0	0	53	64	0	0
p108	12	15	16	31	39	33	56	63	3	11
p110	15	20	19	0	0	0	65	85	0	4
Salmon sperm	0						0			

Rat (208F) and mouse (NIH3T3) cells were transfected with superhelical plasmid DNA as previously described (93). Briefly, 100 ng plasmid DNA was mixed with 10 μg salmon sperm DNA as carrier and coprecipitated using the calcium phosphate technique (178). To each 25 cm^2 flask carrying 1×10^5 recipient cells plated the day before in 5 ml medium, 0.5 ml of the DNA-calcium phosphate-coprecipitate were added. 24 h later the medium was changed with 5 ml non-selective medium (Ham's SF12 for rat 208F and Dulbecco's for mouse NIH3T3) containing 15% fetal calf serum (208F) or 10% donor calf serum (NIH3T3) and incubation continued at 37°C for 24 h. The medium was then changed to selective medium containing 200 μg/ml (208F) or 800 μg/ml (NIH3T3) geneticin (Gibco). When required 1×10^{-6}M dexamethasone or 5×10^{-6}M cadmium chloride were included. The medium was changed every 2-3 days for up to 10 days when colonies were examined and counted using an inverted microscope. Colonies were picked using cloning rings and trypsin after removing the top of the flask with a heated scalpel. The data are derived from the results of six flasks from three separate experiments. Dx, dexamethasone; Cd, Cadmium chloride.

the development of acute myelomonocytic leukaemia through activation of a putative oncogene present on the rearranged chromosome 16.

The powerful ability of enhancer elements contained within retroviral LTRs to direct transcription of adjacent cellular genes is now well established. Thus, the slowly transforming retroviruses which do not contain a transduced oncogene of their own, are capable of activating nearby proto-oncogenes when juxtapositioned by proviral insertion.

The c-*myc* oncogene is a favourite candidate for activation by this mechanism. In mice, leukaemogenesis is associated with proviral insertion of mink cell focus inducing virus (MCF) (133-136), murine leukaemia virus (MuLV) (137), and an intra-cisternal A-particle element (138) at the c-*myc* locus. Similarly in the avian system, naturally occurring cancers are associated with proviral integration of avian leukosis virus (ALV) and chicken syncytial virus (CSV) at the c-myc locus, resulting in the induction of chicken B-cell lymphomas (23, 24, 139-141). In addition feline leukaemia viruses (FeLV) are often found adjacent to c-*myc* in FeLV induced tumours (143). However, LTR activation of c-*myc* expression may not always function directly. Graham *et al* (146) have suggested that alterations in the pvt- 1 locus (approximately 72 kb away from the *myc* gene), in murine T cell lymphomas due to retroviral insertion, disrupts the normal regulation of a putative pvt-1 gene which in turns exerts a long range effect on c-*myc* expression.

C-myc is not the only oncogene activated in neoplastic tissue by proviral insertion. Thus the c-*myb* locus is rearranged in plasmacytoid lymphomas of mice by insertion of Moloney murine leukaemia virus (MoMuLV) (147, 148). C-*erb* transcripts in chickens are truncated by the integration of ALV into a region in the middle of the EGF receptor gene (149) in ALV induced erythroblastosis, and it is likely that precise truncation of the EGF receptor gene is required for oncogenesis (150). Activation of the c-*mos* oncogene in a mouse plasmacytoma also appears to be due to activation by an LTR sequence from an intracisternal A-particle genome (151-153). In addition, the analysis of integration sites of retroviral proviruses associated with specific neoplasia of mice and rats has uncovered further regions of the mammalian genome with oncogenic potential. An analysis of common integration sites of mouse mammary tumour virus (MMTV) in mammary tumours has led to the discovery of the int-1 (154, 155) and int-2 (156) loci. Similarly Guypers *et al* (157) have described a region (Pim-1) of common integration of MCF associated with murine T cell lymphomas, and Silver and Kozak (144) a region (Fis-1) of common integration of Friend MuLV associated with both lymphoid and myeloid leukaemias. In the rat, four separate loci have been implicated in the induction of rat thymic lymphomas by MoMuLV, Mlvi-1, Mlvi-2 and Mlvi-3 (158, 159), and RMo-Int-1 (160).

The strong correlation of tumourigenicity with proviral insertion at well defined oncogenic loci evokes a causative

Figure 4. *Rat 208F cells transformed with* aph *recombinants in the presence of 5 × 10⁻⁶M cadmium chloride. a: RF110-1; b: RF108-1.*

explanation. One such explanation suggests the modulation of proto-oncogene expression by promoter sequences contained within the LTR of the transposed retrovirus. Support comes from studies of oncogene activation directed from integrated free LTRs (145, 161), from a study of the enhancer activity of oncogenic and non-oncogenic avian retroviruses (162), and from the measurement of elevated levels of c-myc in ALV induced bursal lymphomas (23, 24, 139) and in murine T cell lymphomas (137).

Activation of oncogenes by enhancer sequences usually involves the transposition of enhancers from exogenous loci. However, Deschamps *et al* (163) and Renz *et al* (164) have recently identified an endogenous enhancer sequence within the 5′ flanking regions of the c-*fos* gene. Since under normal circumstances c-*fos* does not induce neoplastic transformation of cells, this data suggests that it is not the presence of an enhancer sequence *per se* that is responsible for the activation of a proto-oncogene. The enhancer must also upset the normal regulatory mechanisms controlling transcription. This may therefore be achieved either by the transposition of a foreign enhancer to a proto-oncogene or by affecting the normal function of an endogenous enhancer sequence.

Although it remains difficult to prove, a great deal of circumstantial evidence thereby implicates enhancer sequences in particular those contained within retroviral LTRs as one of the causative agents of oncogene activation in naturally occurring cancers. More direct proof comes from experiments performed on genetically manipulated oncogenes activated in experimentally induced cancers.

8. Enhancers and Experimentally Induced Cancers

The experimental activation of proto-oncogenes by enhancer elements is now well documented and has produced a great deal of information on oncogene function, providing date not readily available by studies on naturally occurring cancers. These experiments have confirmed that it is possible to induce neoplastic transformation in susceptible cells by elevating the level of proto-oncogene expression directed by the enhancer sequences of both SV40 and those contained within retroviral LTRs. Transforming activity has thereby been demonstrated for Moloney murine sarcoma virus (MoMuSV) activation of c-*mos* (9), Harvey murine sarcoma virus (HaMuSV) activation of the normal Harvey ras-1 gene (165), Abelson murine leukaemia virus activation of c-sis (166), and Moloney murine leukaemia virus (MoMuLV) (167), SV40 early promoter (167) and MMTV (168) activation of c-*myc*. In addition, cellular transformation by v-*src* (169) and v-*mos* (170) oncogenes has been rendered hormone responsive by fusion of the activated oncogenes to the LTR of MMTV. Moreover, recent experiments performed by Adams *et al* (171) have provided further important information on the role of c-*myc* in lymphoid neoplasia, demonstrating that deliberate subversion of c-*myc* expression by immunoglobulin heavy chain and kappa light chain enhancer elements is sufficient to induce aggressive leukaemogenesis in transgenic mice.

Experiments performed on the Ha-*ras*-1 oncogene by Spandidos and Wilkie (172) have added the significant observation that neoplastic transformation of early passage rodent cells can be achieved by a single activated oncogene which is both mutated within the coding sequences and produces an elevated level of RNA directed by the combined action of the enhancers of SV40 and MSV. Further experiments performed in our laboratory have attempted to activate expression of the normal Ha-*ras*-1 gene by linkage to the hormone and heavy metal inducible promoter of the human metallothionein IIa gene, in order to study the relationship between the levels of expression of the ras p21 protein and the phenotypic properties of transformed cells through regulation of p21 expression *in vivo*. Recombinant plasmid constructs are described in Figure 3. Plasmid p108 was constructed by replacement of the Ha-ras-1 promoter by the metallothionein promoter sequences fused 61 bp upstream from the Ha-*ras*-1 coding sequences. Plasmid p110 was constructed by insertion of the metallothionein promoter 2 kb upstream from the Ha-ras-1 coding sequences, leaving the Ha-*ras*-1 promoter sequences intact. Plasmids p108 and p110 were transfected in the presence or absence of dexamethasone or cadmium into rat 208F and mouse NIH3T3 cells together with plasmids pAGT1 and pAGN1 carrying the T24 and normal Ha-*ras*-1 genes respectively, linked to the Tn5 encoded aminoglycoside phosphotransferase (aph) gene as a selectable marker (172). The results are shown in Table III. 95% of geneticin resistant colonies obtained by transfection with pAGT1 are morphologically transformed both in rat 208F and mouse NIH3T3 cells. In contrast, pAGN1, expressing the normal Ha-*ras*-1 gene, does not effect transformation in either cell line. p110 is not capable of transforming rat 208F cells either in the presence or absence of hormone or heavy metal stimulation. However, a small but significant number of transformed foci are observed when 3T3 cells are transfected with p110 in the presence of dexamethasone. p108 is, however, capable of transformation of both 208F and 3T3 cells both in the presence or absence of hormone or heavy

metal stimulation. In 3T3 cells at least, the number of transformed foci obtained by transfection with p108 further increases in response to hormone stimulation. Figure 4 shows transfectant rat 208F cell lines obtained by transfection with p110(a) and p108(b) in the presence of 5×10^{-6}M cadmium chloride. The morphology of p110 transfectant cell lines RF110-1(a) remains essentially normal whereas that of p108 transfectant RF 108-1(b) consists of transformed refractile pebble-shaped cells which grow in a disorientated manner and are not contact inhibited. The above data confirms the previous observation by Chang *et al* (165), that the normal Ha-*ras*-1 oncogene is capable of morphological transformation when activated by an enhancer element. The differences in behaviour between rat 208F and mouse NIH3T3 cells does however suggest that the threshold level of tolerance to p21 protein may vary from cell to cell. The data also shows that transformation mediated via an inducible Ha-*ras*-1 gene can be regulated by exogenously added glucocorticoid hormones or by heavy metal ions. By *in vivo* regulation of *ras* p21 protein, it should now be possible to study in greater detail than before the relationship between the levels of expression of p21 protein and the phenotypic properties of transformed cells.

9. Concluding Remarks

Disruption of the control mechanisms which regulate expression of proto-oncogenes clearly plays a role in the development of some cancers. It is not surprising then to learn that powerful promoter elements in the form of enhancer sequences are often found transposed to proto-oncogenes during tumourigenesis. The evidence thereby implicating enhancer elements in the genesis of certain cancers is compelling and comes from scrutiny of both naturally occurring and experimentally induced cancers. Nonetheless, the contribution made by enhancers is likely to vary. Overexpression of a proto-oncogene may often be the result. However, it is conceivable that in some cases altered temporal expression may occur resulting in activation of a proto-oncogene at an abnormal stage of the cell cycle or during cellular differentiation. It is possible that the degree of host cell specificity of enhancer function may be inextricably linked with this process. Whatever the mechanism of proto-oncogene activation, it is evident that the study of transcriptional enhancers has contributed significantly to our understanding of the mechanisms of carcinogenesis at the molecular level.

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