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Different methods to follow protein–protein contacts in a multi-subunit system*

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Abstract

Protein–protein interactions among nearest neighbours or between two distant proteins play major role in the development and spatiation of an organism To understand the basic rules that govern these interactions various physical approaches are taken which are inadequate to describe the *in-vivo* situation Generation of a phenotypic mutation in a protein and then looking for its suppressor may allow to map the interacting partners without perturbing the system very much from its original phenotype This review aims to list various such studies and addresses the future course that can be persued

Keywords Contact sites, protein–protein interactions, crosslinking spectroscopy genetic suppression

1 Introduction

Specific protein–protein and protein–DNA interactions govern the regulation of all cellular processes which constitute a living cell The basis of all essential biological functions is the ability of the cell to elicit the required cellular response by specific molecular interactions For example, replication apparatus, transcription machinery, translation complexes, enzyme assemblies, receptor–ligand interactions and transport pathways all mediate their respective functions by crosstalk between specific macromolecules Protein–protein interaction is an important subset of a variety of interactions constituting biological processes

The transcription apparatus in *Escherichia coli* consists of a DNA-dependent RNA polymerase (RNAP) which is a multi-subunit enzyme with subunit composition of $\alpha_2\beta\beta'\omega\sigma$ Interactions within and between the subunits result in the formation of catalytically competent enzyme capable of promoter-specific interaction of transcription Moreover, specific interactions between RNAP and transcription activators or DNA finetune the transcription apparatus to meet the myriad cellular needs Thus, an insight into protein–protein interactions provides a basis for understanding the highly ordered and regulated process of transcription in *E coli*

2 Methods to study protein–protein interactions

A number of biochemical approaches have been used to study interactions between proteins Many interactions in multi-subunit complexes have been elucidated using methods like

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crosslinking, protein footprinting, protease sensitivity profiles and mutagenesis These methods, in combination with *in-vivo* approaches like suppression analysis have paved the way in understanding the functional relevance of protein–protein interactions in a multitude of cellular processes

Chemical crosslinking has been one of the oldest methods to probe direct contact between two proteins using a large number of reagents [1 2] The *E coli* ribosome is a complex multiprotein assembly comprising 52 proteins and three RNA molecules Chang and Flake[3] used a group of bifunctional sulfhydryl reagents, the phenylene dimaleimides which crosslink a pair of 30S ribosomal proteins with remarkable selectivity Results of their study indicated specific crosslinking between proteins S18 and S21 and that the crosslinking involves specific sulfhydryl groups located on each of these proteins They also found that crosslinking occurred only in 30S subunit and not with the two proteins free in solution Their studies suggested that S18 and S21 are probably located adjacent to each other and possibly on the surface of 30S subunit as the crosslinking of the two proteins was found to occur through exposed sulfhydryl groups Chemical crosslinking using bifunctional reagents was also used to elucidate the subunit interactions with the *E coli* RNAP [4] Their investigation showed $\beta\beta'$ crosslinked product to be the major band on analysis by SDS–polyacrylamide electrophoresis thus suggesting the area of contact between the two subunits to be large McMahan and Burgess[5] optimised the use of aryl azide crosslinkers to study interactions within the subunits of *E coli* RNAP and showed α–σ interaction and that contact region on α-subunit is the C-terminal domain Chen *et al* [6] used protein–protein photo crosslinking to determine direct contact between the *E coli* RNAP and the activation region of catabolite gene activator protein (CAP) A photoactivable crosslinking agent was targeted to a specific residue of CAP followed by UV irradiation of the ternary complex The transfer of the radiolabel from the crosslinker to the point of contact on the ternary complex followed by subsequent cleavage was used to identify CAP–RNAP contact region Results suggested α-subunit C-terminal domain to be in direct contact with the activation region of CAP σ^{54} interaction with a transcriptional activator C4-dicarboxylic acid transport protein D (DCTD) from *Rhizobium meliloti* and RNAP was elucidated using bifunctional crosslinking reagents [7] Their investigation indicated that σ^{54}-dependent activator DCTD may engage both σ^{54}- and β-subunit of RNAP to activate transcription Direct protein–protein interactions can also be monitored by a method analogous to DNA footprinting, a technique wherein the DNA substrate is end-labelled with ^{32}P and subjected to chemical cleavage or enzymatic digestion The resulting fragments can then be resolved by electrophoresis In the presence of a protein molecule which can interact with the DNA substrate, a 'footprint' or 'shadow' results in the region of DNA–protein contact as this area is occluded from cleavage [8] The same principle can be extended to study interacting regions between two proteins In this 'protein footprinting' method, end-labelling of the protein is achieved by placing a short linker sequence encoding a peptide which is a substrate for a protein kinase at one end of the gene and subsequently the protein is end-labelled with ^{32}P ATP The labelled protein, after being mixed with an interacting protein, is subjected to limited proteolysis The products are then analysed by SDS–polyacrylamide gel electrophoresis and autoradiography [9] Zhong *et al* [9] have illustrated the efficacy of their method by demonstrating the interaction of myoglobin and an antimyoglobin monoclonal antibody using the same technique Nagai and Shimamoto[10] investigated the *E coli* σ^{70}-core RNAP interactions and their studies indicated

that subdomains 2 2 and 2 4 are clearly protected in holoenzyme, suggesting their direct interaction with core RNAP Besides these regions, domain 1 1 and segment of domain 3 also were found to directly interact with core RNAP Interaction of segments of the T4 late genes σ factor, gp55, with *E coli* RNAP was analysed using protein footprinting and regions corresponding to subdomains 2 1 and 2 2 were found to form the σ-core enzyme interface [11]

The protein complexes in a protein footprinting reaction can also be cleaved[12] by radicals generated by metal/H_2O_2 and is more versatile than proteolytic enzymes as the latter cleave only a limited number of sites in the protein Moreover, proteolytic resistance varies from protein to protein Fe-EDTA probes were found to be nonspecific chemical proteases[13] and replacing proteolytic enzymes with them has greatly enhanced the resolution of the footprints Fe-BABE [Fe(S)-1-(*p*-bromoacetamidobenzyl)EDTA], one such Fe-EDTA probe, can be tethered to the Cys residues and it can cleave within 12 Å of its attachment site [14] The mode of cleavage is illustrated in Fig 1 This method of probing protein–protein interactions has a much higher resolution as the pattern of cleaved fragments gives a footprint which represents the interface between the two proteins Resolution of crosslinking studies is limited to studying the proximity of two proteins with respect to each other and does not extend to mapping the regions of contact Fe-BABE has been effectively used as a chemical protease after conjugation with *E coli* σ^{70} to map σ^{70}-core RNAP contact sites On the β-subunit, the σ^{70} contact sites have been mapped onto two regions, (i) between amino acids 383 and 584, including conserved region C and Rif region, and (ii) between residues 854 and 1022, including conserved region G which includes ppGpp-binding site and the segment forming the catalytic centre On the β'-subunit, the σ^{70} contact sites have been mapped to conserved regions C and D [15]

Mutagenesis is another powerful tool which has been employed to identify critical amino-acid residues constituting interacting surfaces between proteins Preliminary identification of functionally relevant segments of a polypeptide can be achieved by deletion analysis In this method, a small number of amino acids is progressively deleted from C- or N-terminal regions (or even internal fragments) and the mutants can then be assayed *in vitro* for their ability to mediate protein–protein interaction Alternatively, functional relevance of the mutation can be assessed by its phenotype Site-directed mutagenesis can then be employed to pinpoint functionally important amino acids Regions in N-terminal domain of α-subunit of *E coli* RNAP interacting with β- and β'-subunits were identified using insertion mutagenesis and amino-acid substitutions within the α-subunit [16 17] Mapping of α–β subunit contact sites in *E coli* RNAP was also facilitated by the use of truncated fragments of β-subunit and proteolysis of $\alpha_2\beta$ complex and free β-subunit [18]

3 Genetic suppression

Suppressor mutations are those which occur at a second site, at a distance from the original mutation and by themselves may not have a phenotype but in the presence of the original mutation can restore the phenotype to that of wild type and hence the term 'suppressor' Thus, suppressor mutants are those that ameliorate the primary mutant phenotype Broadly, genetic suppressors can be intragenic wherein the second-site mutation is within the same gene where

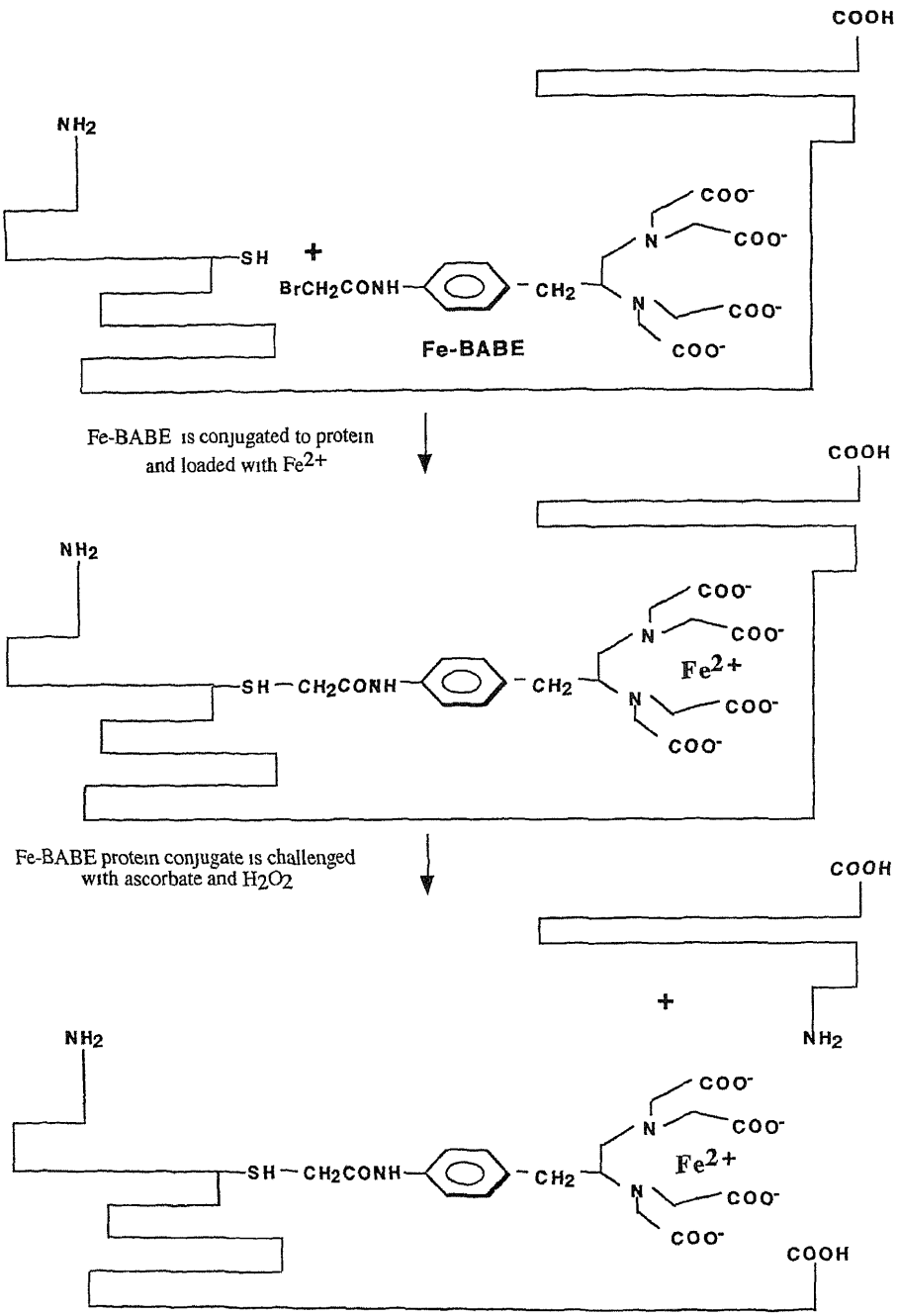

FIG 1 An outline of cleavage of the peptide bond by Fe-BABE

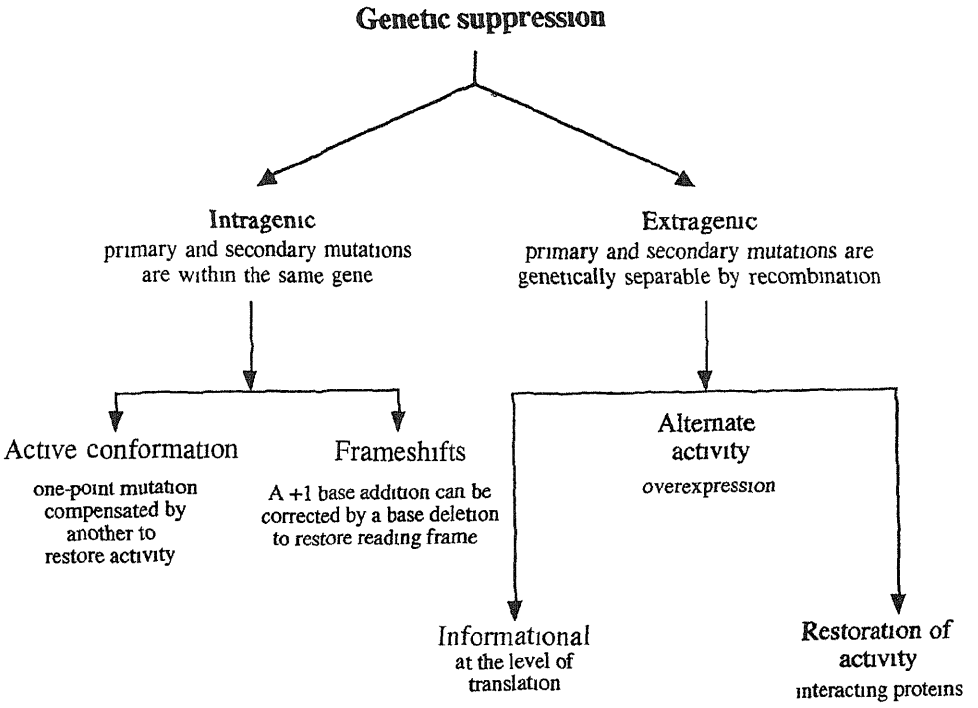

FIG 2 A schematic representation of different mechanisms by which genetic suppression could occur

the original mutation is present or extragenic (also called intergenic) wherein the second mutation resides in a locus distinct from the original mutation [19 20] An outline of the mechanisms of genetic suppression is illustrated in Fig 2

3 1 *Intragenic suppression*

An intragenic suppressor balances the effect of an original mutation by occurring within the same gene The suppressor may be a true revertant or change in the same codon as the primary mutation thus resulting in a less detrimental residue in that position or by a change in another codon within the polypeptide Intragenic suppressors can be distinguished from extragenic ones by linkage analysis Intragenic suppressors restore the primary defect through a number of mechanisms

3 1 1 *Active conformation*

Helinsky and Yanofsky[21] described a mutation in *E coli* tryptophan synthase A protein at position 210 where a Gly to Glu substitution inactivated the enzyme Reversion analysis of this mutant revealed a second-site mutation within the same gene, a Try to Cys substitution at position 174 which had the ability to restore enzymatic activity to the previously inactive mutant Neither mutation, in isolation, was able to confer enzymatic activity Likewise, a muta-

Intragenic suppressor analysis Prediction of interacting domains within a protein

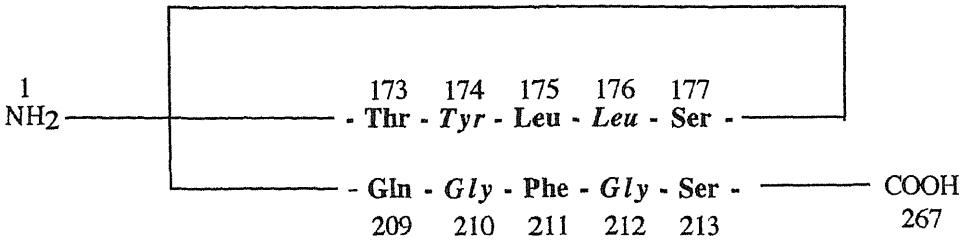

FIG 3 Possible contact regions within the tryptophan synthase A molecule as suggested by genetic evidence (Yanofsky *et al* [27])

tion at position 176 was compensated by a mutation at position 212 [22] This led Yanofsky and coworkers to speculate that region 210–212 of the protein may interact with region 174–176 of the same protein The likely mode of suppression is through compensation of a second mutation in the interacting region thus restoring wild-type activity to the enzyme (Fig 3) Similarly, Jinks[23] isolated suppressor mutations in T4 bacteriophage gene*h* which encodes a protein involved in phage adsorption, mapping very close to the site of original mutation Here too, the mechanism of suppression could be similar to that of tryptophan synthase A Apart from proteins, tRNA molecules can also undergo second-site reversions thus providing an insight into the conformation and interaction of tRNA molecules with other molecules, *in vivo*, as described below

The tyrosine tRNA molecule can be mutated in its anticodon region such that it now possesses the ability to recognise the nonsense triplet UAG and can insert a tyrosine there Mutations that abolish the suppression activity of the tRNA can now be isolated Second-site mutations of these nonfunctional tRNA suppressors which enable them to regain their suppressor phenotype were isolated and an analysis of the mutants revealed that bases 2 and 80 of the tRNA molecule are in contact as was predicted by the clover leaf model [24 25] The Tn10-encoded metal–tetracycline/H^+ antiporter of *E coli* (tetA gene product) has a critical Asp residue at position 285 which is essential for its antiporter activity Mutation of this residue renders it inactive and its activity was restored by a second-site mutation, A220D The acidic residue at position 285 was compensated for by an acidic amino acid at postion 220 [26] Bowers and Dombroski[27] isolated a suppressor of σ^{70} (the vegetative sigma factor of *E coli* RNAP) mutant which was proficient in holoenzyme formation but was severely impaired in initiation of promoter-specific transcription The primary mutation, I53A, resides in the 1 1 subdomain which modulates DNA-binding by domains 2 and 4 when σ^{70} is in the free form I53A phenotype, as a result of its inability to undergo critical conformational change necessary to expose the DNA-binding domains, thus conferring promoter specificity, was suppressible by a five-amino-acid truncation at the C-terminal region of σ^{70} The suppressor functions by loosening the intramolecular contact between the N- and C-terminal domains, after association with the core enzyme, thus exposing the DNA-binding regions of the protein accessible in order to initiate promoter-specific transcription

3 1 2 *Frameshifts*

The addition or deletion of bases in multiples other than three will result in the alteration of reading frame of the genetic code, thus leading to the formation of prematurely terminated or an altered protein during translation In principle, a second compensatory mutation in the same gene can restore the reading frame, thus rendering the protein active For example, A-1 frameshift mutation which is deletion of a single base can be compensated by the addition of a base not too far away from the site of deletion, thus restoring the reading frame It is imperative that the addition of a base be close to the –1 frameshift as this would minimise the number of residues which have been altered If the small number of residues so altered does not lie in a critical segment of the protein, then the activity of the mutant protein could be restored Such a case has been observed in phage T4 lysozyme A-1 frameshift was compensated by a +1 frameshift and the resultant protein was active as it differed in the sequence of only five amino-acid residue from the wild-type protein before the correct reading frame was restored [28] The major contribution of this study was verification of the proposal by Crick[29] that translation of genetic message is initiated at a given point and proceeds by sequential reading of defined group of nucleotides Thus, the intragenic second-site revertants provided a tool to confirm a fundamental characteristic of the genetic code

3 2 *Extra(inter)genic suppression*

A second possibility of obtaining a suppressor is that the restoring mutation is in a genetically distinct locus from the original mutation Such second-site mutations which are present in a distinct second functional unit of genetic material can be genetically separated by recombination from the original mutation that they suppress and are termed 'inter-' or 'extragenic' suppressors They use a number of mechanisms to restore or compensate for the original defect

3 2 1 *Direct or informational suppression*

In this form, the mRNA containing the defect is mistranslated at the region of the mutation by the translation apparatus A mutation in any of the components of the translation apparatus which decreases the fidelity of the system will tend to misread the original defect, thus rendering the protein active again Depending on the nature of the primary mutation, the mechanism of suppression varies One mode of direct suppression which is more specific than the kind executed by the ribosomal protein mutations is by suppressor tRNAs These molecules harbour a mutation in the anticodon region thus enabling them to misread Consequently, these tRNAs can no longer translate their original codons However, normal translation of the original codons remains unimpaired as most codons have multiple tRNAs or multiple identical copies of the tRNAs Nonsense codons are chain-terminating triplets which are of three kinds UAG (amber), UAA (ochre) and UGA (opal), amber suppressors have an anticodon CUA [30] Alternatively, mutations in regions other than the anticodons of the tRNAs can also lead to suppression [20] An interesting case of informational suppression was reported by Riddle and Carbon [31] They observed that strains of *Salmonella typhimurium* carrying a frameshift suppressor *sufD* encoded a glycine tRNA having a quadruplet CCCC as its anticodon Such an anticodon enabled the *sufD* to effectively recognise +1 frameshifts near or in CCC codon in histidinol dehydrogenase gene and restore the correct reading frame An alternative means of restoration of frameshifts was discovered in the yeast mitochondrial serine tRNA where the anticodon loop

was unaltered but its stem harboured a base substitution This altered conformation of tRNA as a result of the mutation enabled it to read a four-base codon thus restoring the frameshift [32–34]

Besides suppression by tRNA molecules, the protein components of the ribosomes and antibiotics like streptomycin can ameliorate mutations in mRNA during the process of translation The *strA* mutants of *E coli* are streptomycin resistant and the mutation maps to one of the structural genes of the 30S ribosomal subunit protein In the presence of this mutation, a high measure of restriction of nonsense mutations as well as missense mutations was observed The addition of streptomycin alleviated this restriction by enhancing the ambiguity intrinsic to the code Thus, *strA* mutations and streptomycin are mutually antagonistic It was proposed that the antibiotic produces an alteration at the codon–anticodon recognition step Effects of mutations in another locus *ram* (ribosomal ambiguity) defining a 30S component were found to be similar to that of addition of streptomycin *In vivo*, this mutation was able to restore the suppressor activity of nonsense and missense suppressors [35]

3 2 2 *Restoration of protein activity*

Another mechanism of suppression of mutations in proteins which could be envisaged is that which alters the intracellular environment such that now the mutant protein is rendered functional in the new milieu These suppressors provide valuable information on the effect of cellular environment on the enzymatic function of a protein Osmotic remedial mutants[36] resulted in a mutation *eth-1* in *Neurospora* which had multiple phenotypes This mutant was ethionine (an unnatural amino-acid analog) resistant, was unable to grow in minimal medium at 36°C and showed repression of several enzymes by methionine This mutant phenotype was suppressed by merely growing the strain in a high osmolar medium Remediation of this phenotype was best explained by the hypothesis that the nonfunctional proteins can be rendered active by high osmotic pressure

Reconstruction of enzyme conformation to restore activity has proved to be an important means of genetic suppression Complex structures like ribosomes contain a large number of protein–protein interactions which are critical for the translation machinery Compensatory mutations restoring functionality of original mutations are seen in the ribosomal assembly [37] Similar compensatory mutations in the *lac*-repressor gene in response to mutations in the *lac*-operator region have been used to delineate regions of the suppressor which are involved in interactions with the operator region [38] Ohashi *et al* [39] isolated a suppressor of a temperature-sensitive σ^H (the sporulation sigma factor of *Bacillus subtilis*) mapping in the β-subunit of RNAP and their studies revealed that this interaction with β-subunit prevented the degradation of σ^H at the nonpermissive temperature

3 2 3 *Alternate protein activity*

Recombination in *E coli* involves a large number of genes Mutation in two genes *recB* and *recC* results in impairment of an ATP-dependent DNase activity and hence in the recombination Second-site revertants of *recB* confers an ATP-independent DNase activity unlike the one associated with *recB* gene product Thus, the suppressor mutation is in a locus which has effectively increased the expression of a DNase which is pre-existing, thus compensating for the deficiency in *recB*$^-$ strain [40] Many such compensatory mutants have provided an alternative

mechanism to restore the defect of the original mutation [20] *E coli* has three single-stranded DNA-specific exonucleases RecJ, exonuclease I and VII A triple mutant of these enzymes is highly UV-sensitive probably because of their inability to repair lesions that block DNA replication Multi-copy suppression analysis of this triple mutant yielded a single gene encoding RNase T, whose normal function is removal of terminal adenine residue from 3'-end of immature tRNA This study revealed an alternative DNase activity of RNase T in the triple mutant background thus compensating for their activity [41]

3 3 *Suppression analysis as a tool to probe protein–protein interactions*

A second-site mutation, be it intra- or intergenic, can restore functionality to a dysfunctional gene As already described, a number of mechanisms are operational and have contributed substantially to the understanding of gene structure and function Genetic suppression has been a powerful tool in exploring protein–protein interactions between components in multi-protein assemblies and between domains of the same protein There are two ways in which a mutant phenotype can be restored to a wild-type phenotype First, mutations rendering a protein dysfunctional by virtue of its inability to now interact with another protein are restored by a corresponding mutation on the interacting surface of the second protein which is now able to accommodate the original mutation Secondly, the restoring mutation on the second protein may not be located in the interacting surface, but in a region which alters the conformation of the protein in a manner which enables it now to interact with the first mutant protein Most often, former class of suppressors, be they inter- or intragenic, are allele-specific That is, the suppressor mutation will be able to suppress only the original mutant allele and no other allele of that gene Adjunct to this is that suppressors, indicative of direct physical contact between two proteins or between two regions within the same protein, tend to cluster in a small region rather than be distributed in a number of segments Hence, allele-specific suppression has been used to define contacting regions in a wide variety of protein–protein interactions However, suppression analysis may not always indicate distance geometry [42]

Wu *et al* [43] studied the bacteriophage lambda-encoded integrase, a site-specific recombinase using second-site reversion analysis Suppressor of the primary mutation P 243L resulted in an intragenic suppressor E218K This secondary mutant was not allele-specific as it could suppress T270I E218K probably acts by improving the inherent activity of the integrase rather than by a direct compensation for the defect caused by the primary mutation The glutamic acid-to-lysine substitution increased the net positive charge on the protein and the compensatory action of this suppressor may be by improving the interaction of the integrase with DNA The *cheC* gene of *E coli* encodes a protein which is involved in regulating the flagellar rotation during chemotactic response *cheC* gene product may be a structural component of the flagellum In an effort to identify the interaction of *cheC* with other components of chemotactic behaviour, Parkinson and Parker[44] used second-site reversion analysis of *cheC* mutants The intergenic suppressors of *cheC* are allele-specific and were localised to *cheZ* gene Mutations in *cheZ* increase the tumbling frequency of the bacterium while those in *cheC* have the opposite effect It is the interaction between these two gene products that sets the spontaneous tumbling rate of the cell The eukaryotic cytoskeleton is a complex, dynamic structure consisting of a large number of interactions Although individual components of the cytoskeleton have been identified, functional associations and their relevance have not been easy to demonstrate

In-vitro methods using drugs and microinjected antibodies have proved useful but limited in understanding the functional interactions within the cytoskeleton Genetic analysis using second-site revertants was used as an alternative way Adams *et al* [45] used temperature-sensitive mutations of an essential actin gene of *Saccharomyces cerevisiae* to isolate suppressors of the temperature-sensitive phenotype The true second-site mutants were mapped onto a locus *sac6* Neither mutation in isolation had wild-type phenotype *sac6* was shown onto encode an actin-binding protein A compensatory change in *sac6*, in response to the primary temperature-sensitive mutation in actin gene, was able to restore normal cytoskeleton organisation indicating that these two proteins do interact *in vivo*

Genetic suppression has proved to be a very efficacious tool in understanding and dissecting functional interactions in multi-subunit complexes *E coli* F_0-F_1 ATP synthase synthesises ATP coupled with an electrochemical gradient of protons F_1 unit is a multi-complex assembly comprising α, β, γ, δ and ε subunits and the F_0, of a, b and c subunits Missense mutations in a-subunit impairs proton translocations Working on the premise that if two or more sites in a protein participate in critical interaction, mutations in one interacting region can be compensated for by a corresponding change in the other position Hartzog and Cain[46] isolated intragenic suppressors of a-subunit in order to pinpoint the determinants involved in protein–protein interactions The a-subunit mutation G218K inhibits F_0-F_1 ATP synthase activity to below detection limit and is suppressed by H245G This suggests that these two segments of a-subunit are spatially in close proximity In a similar study of F_0-F_1 ATP synthase, intergenic suppressors of a frameshift mutation in the γ-subunit were isolated by Beukelaer *et al* [47] in order to study the conformational transmission between the β- and γ-subunits The primary mutation was γ-frameshift resulting in altering the last 16 amino-acid residues at the carboxyl terminus Such a mutant was unable to grow by oxidative phosphorylation and exhibited very low ATPase activity This defect was suppressed by either R52C with or without a second mutation V77A in the β-subunit or by G150D in the β-subunit of the enzyme The carboxyl terminus of wild-type γ-subunit is an α-helix while the frameshift mutation is a β-sheet and earlier work suggested interactions between this region of β-and γ-subunits The β-barrel domain of β-subunit contains the suppressor mutation R52C and hence compensated for the altered β–γ interactions in the primary mutant The mutant G150D may be suppressing through a different mechanism which affects conformational transmission, rather than by direct compensation of the primary mutant

The transcription apparatus is a multi-complex system comprising a multi-subunit RNAP and in eukaryotes, a multitude of transcription factors Three forms of RNAP exist in eukaryotic cells, namely, RNAP A, B and C (also called I, II and III) and each transcribes a distinct set of genes RNAP I catalyses the synthesis of rRNA, RNAP II transcribes mRNAs and RNAP III synthesises tRNAs and 5S RNAs [48] Genetic analysis has provided much insight into the structural and functional organisation of this complex machinery Suppression analysis has been used extensively to study protein–protein interaction between the subunits of *Saccharomyces cerevisiae* RNAP II Nonet and Young[49] employed suppressor analysis to investigate the role of a heptapeptide repeat found in the C-terminal domain (CTD) of the largest subunit (RPB1) of RNAP II This repeat can be phosphorylated and its phosphorylation status regulates the transition from initiation to elongation complex [50] Mutations in this repeat results in temperature- or cold-sensitive phenotypes These conditional mutants were used by Nonet and

Young[49] to isolate the second-site revertants A class of intragenic suppressors mapping to a conserved region, homology box H of RPB1 indicated that this region interacts with the heptapeptide consisting of pro-thr-ser-pro-ser-tyr-ser repeats in the C-terminal domain Allele-specific extragenic suppressors led to the identification of a new locus SRB2 gene which encodes a component of the transcription apparatus Martin *et al*[51] used a similar strategy to define interactive domains of the two large subunits of *Saccharomyces cerevisiae* RNAP II A temperature-sensitive allele of RPB1 was used as a starting point to isolate second-site revertants on RPB2 gene Two features of RPB2 suppressors, allele-specificity and clustering of mutations in region I, provide evidence of local interactions between region I of RPB1 and region H of RPB2 Likewise, extragenic allele-specific suppressors of a temperature-sensitive mutation in RPA 190 encoding the largest subunit of RNAP I of *Saccharomyces cerevisiae* was found to map onto the second largest subunit A135, in a region encompassing a putative zinc-binding domain The primary mutation in RPA190 also localises within a putative zinc-binding domain [52] From the above study, the authors propose direct interaction of the two putative zinc-binding domains Martin *et al*[51] isolated suppressors of a conditional lethal mutation in the largest subunit of RNAP II of *Drosophila* in an effort to identify the components of transcription machinery In this screen, suppressors mapped to a locus encoding the second largest subunit of RNAP which was hitherto unidentified Allele-specific nature of this second-site mutations suggested direct interaction of this gene product with the largest subunit of RNAPII

The E coli RNAP has also been an attractive multi-subunit system amenable to genetic analysis which has provided new insights into functional interactions within the subunits of the enzyme and with factors interacting with polymerase The NusA protein is a modulator of transcription termination and antitermination and exerts its effects through interactions with RNAP Conditional lethal *nusA* allele, G181D, was used as primary mutant to isolate second-site revertants Sequence analysis of intragenic suppressors revealed a D84Y substitution in the NusA protein, suggesting the close proximity of D84 to G181 in its the tertiary structure Extragenic suppressors mapped onto *rpoC* which encode the β'-subunit of RNAP indicating genetic interaction of NusA with β'-subunit In this case, however, direct interaction may not be the mode of suppression as evidenced by antibody against this region of NusA which does not prevent β'-NusA association [53]

Interactions within the β-subunit of *E coli* RNAP have been of great interest as the 1342 amino acids containing the polypeptide harbour the substrate-binding site [54] Singer *et al* [55] dissected the rifampicin-binding site on β-subunit using genetic suppression (Fig 1 outlines the conserved domains of the two large subunits of *E coli* RNAP) This site comprises two regions, clusters I and III, and a single primary mutation in cluster III confers rifampicin resistance and is both cold as well as temperature-sensitive Using these properties, second-site intragenic suppressors in cluster I region were isolated and characterised to be allele-specific Functional restoration by the cluster I mutants indicates that these two regions interact and contribute to the formation of rifampicin-binding site In a continued effort to understand regions of interaction within the β-subunit, intragenic allele-specific suppressors of elongation-deficient inviable *rpoB* alleles were isolated [56] One of the primary mutations conferring severe elongation and termination defects is located in region Rif cluster II, a part of a highly conserved segment, region D The corresponding suppressors mapped onto region B The second primary β-subunit mutant used as a starting point was A676V, located in conserved region E

The allele-specific suppressors of this mutant fall within the conserved region H Neither of the primary mutants nor their suppressors, in isolation, could support growth, thus augmenting the hypothesis that interactions among region B–region D and region E–region H within the β-subunit are necessary for functional integrity of the polypeptide

4 Conclusions

Genetic suppression has been used extensively to understand protein–protein interactions and their functional relevance in a wide variety of cellular processes However, this method is not infallible as demonstrated by Meunier and Rich [42] The authors isolated assembly- and activity-deficient mutants of yeast cytochrome *c* oxidase subunits I and II and their revertants to assess the prediction of distances between them Their study indicates that second-site mutations could occur at distances as far as 30Å from the primary mutations Hence, a combination of biochemical and genetic evidences could prove to be more illustrative in correlating structure with function than either method in isolation

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