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EDITED BY

G. H. BOURNE J. F. DANIELLI

ASSISTANT EDITOR

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Chromosomal Localization of Protooncogenes

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I. Introduction

During the last several years, the general area of tumor biology coalesced into a tighter, more unified discipline when it was discovered that the transforming activity of retroviruses was due to a series of cellular genes that had been acquired by retrovirus genomes (Bishop and Varmus, 1982). These cellular-derived sequences apparently acquired their transforming activity either by being placed under the control of an active viral transcriptional promoter or by acquiring limited mutational events which altered their activity or interaction with other molecules. (For a review on retrovirus oncogenes, see Bishop and Varmus, 1982; Bishop, 1983.) This unification has gained additional importance with the realization that at least some of these cellular genes have been implicated in the formation of several human cancers, as judged by *in vitro* transfection assays (Bishop, 1983).

During 1982, this fast-moving area of tumor biology began to merge into yet another discipline, tumor cytology. It has been known for many years that certain tumors have specific chromosomal structural abnormalities. The Philadelphia chromosome associated with chronic myelogenous leukemia (CML) (Sandberg,

1980; Mitelman and Levan, 1981) and the translocation t(8;14) associated with Burkitt's lymphoma (Yunis, 1981; Rowley, 1982) are among the best examples, but several other abnormalities frequently associated with a variety of human neoplasia also have been described (Sandberg, 1980; Yunis, 1983). Although these structural changes were deemed important, it was not until the chromosomal location of several oncogenes was determined that the real molecular significance became apparent. It now appears that many of the genes associated with animal tumors are located near specific translocations in human cancers.

The following discussion will define and briefly discuss the concept of protooncogenes and will then describe the studies by which these genes have been located in a variety of species, with a marked emphasis on man. Although the theme of this article is pointing out the close proximity of protooncogenes with tumor-associated chromosomal anomalies, it should be stated that no protooncogene has definitively been shown to be converted to an oncogene as a direct consequence of a translocation. In fact, the complex nature of most human tumors would suggest that multiple events are required and that gross chromosomal alterations play a significant but limited role in the formation of some tumors.

II. What Are Protooncogenes?

During the early 1970s it was discovered that most retrovirus strains were composed of two genome types: one, a replication-competent virus which would not cause an actue disease; and the other, a replication-defective genome that contained sequences unrelated to the nondefective viral genome. Through the use of temperature-sensitive (ts) and deletion mutants, it was conclusively shown that these replacement sequences were responsible for rapidly transforming cells *in vitro* and *in vivo* and were aptly called oncogenes (Linial and Blair, 1982). An examination of various retrovirus strains associated with a variety of animals has uncovered approximately 20 distinct oncogenes, although recent evaluation of nucleic acid sequences and deduced protein sequences has suggested that several of these genes have evolved from common progenitors (Levinson *et al.*, 1981).

During this period, it was also discovered that normal, uninfected cells contained sequences related to the viral oncogenes (Stehelin *et al.*, 1976). These cellular sequences were later shown to be distinct from endogenous retrovirus genomes (Padgett *et al.*, 1977; Tereba *et al.*, 1979), were present in most species (Shilo and Weinberg, 1981), were typical eukaryotic genes containing from none to many introns (Bishop, 1983), and were normally expressed in a variety of tissues (Muller *et al.*, 1982). Manipulation of some of these cellular sequences showed that they had the potential for causing oncogenesis when placed in the right environment (Osharsson *et al.*, 1980; Defeo *et al.*, 1981; Chang *et al.*,

1982). These sequences were thus termed protooncogenes with a "c" for cellular prefixing the three-letter code for the viral or v-*onc* genes.

Recently, oncogenesis by cellular genes has been successfully assayed by transfecting tumor DNA into NIH-3T3 cells. This procedure has resulted in an additional set of oncogenes overlapping their corresponding normal protooncogene counterparts. In this assay, the *ras* gene family has been implicated in many solid tumors, although several transforming genes associated with hematopoietic cancers have been detected which show no homology to sequences incorporated into known retrovirus genomes.

Of particular importance to speculations about the normal function of these protooncogenes is their highly conserved nature. All vertebrates examined contain an array of these genes, and some protooncogenes such as c-*myc* and c-*src* have been detected by nucleic acid hybridization in the DNA from *Drosophila* and the worm *Caenorhabditis elegans* (Shilo and Weinberg, 1981). Their omnipresent and highly conserved nature implies that these genes are important for fundamental cell functions. Indeed, the expression of several protooncogenes in a variety of species has been detected in most cell types examined (Muller *et al.*, 1982). Other protooncogenes appear to be expressed in specific cell types and at select stages of differentiation (Muller *et al.*, 1982; Chen, 1980; Westin *et al.*, 1982; Rosson and Tereba, 1983). This selectivity may account in part for the cell-type specificity that certain v-*onc* genes display. In support of these genes having fundamental roles in the growth of cells, it has been shown by deduced amino acid sequence that B*lym* shares a domain of partial homology with the transferrin gene family (Goubin *et al.*, 1983); v-*sis* shows an extensive homology with the platelet-derived growth factor (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983) and v-*erb*-B corresponds to a truncated version of the epidermal growth factor receptor (Ullrich *et al.*, 1984). In any event, a knowledge as to which normal functions these gene products perform will greatly help our understanding of their role in oncogenesis.

III. Chromosomal Localization of Unique Gene Sequences

Localization of single copy gene sequences to distinct chromosomes has been accomplished by a variety of techniques. Each procedure has its advantages and disadvantages and is dependent to some extent on the species being examined and the detail that is desired. As in most investigations, it is desirable to use at least two approaches, if possible, since all of these procedures rely on somewhat subjective evaluations of chromosomes that are sometimes prone to artifacts. This section is designed to acquaint the reader with the various techniques utilized to localize distinct protooncogenes. The procedures are presented in sufficient detail to make clear the difficulties of each technique and their advan-

tages and disadvantages. Detailed techniques should, however, be obtained from the cited references.

A. Chromosome Fractionation

Although chromosome fractionation has severe limitations regarding resolution and the type of cells that are suitable for this procedure, it has been successfully used and shows promise as a first step in performing other techniques. This procedure requires a cell line with a reasonably short generation time or normal cells that can be more or less induced into synchronous growth such as hematopoietic B and T cells. Early experiments utilized large-scale fractionation in sucrose gradients (Padgett *et al.*, 1977), a technique that is effective at separating chicken chromosomes into several enriched chromosome fractions due to the wide distribution in chromosome size. However, this procedure would be unsuitable for human and mouse chromosomes. Further separation of chromosomes has been accomplished using a fluorescent activated cell sorter (FACS) which separates chromosomes mainly on the basis of DNA content. The degree of separation is dependent upon which fluorescent DNA binding dye is used, the compactness and aggregation of the chromosomes, and whether one or two lasers are employed. Current techniques can separate all but one of the first nine chicken chromosomes (Stubblefield and Oro, 1982) and 17 of the 23 human chromosomes (Gray *et al.*, 1979; Dean and Pinkel, 1978). In addition, enough DNA can be obtained from the separated chromosomes to perform Southern blot analyses so as to localize specific unique gene sequences to distinct chromosomes and to generate DNA libraries of specific chromosomes.

In general, while this technique has been successful, it has limitations and requires an expensive FACS to obtain resolution of distinct chromosomes. It does have potential as an initial step in the generation of somatic cell hybrids containing specific heterologous chromosomes and as an initial sorting step for *in situ* hybridization techniques.

B. Somatic Cell Hybrids

Somatic cell hybrids have been used extensively in the localization of many human genes, including several protooncogenes. The technique requires several cell clones containing a few defined heterologous chromosomes. This is easily accomplished as heterologous cells fused with polyethylene glycol randomly eliminate chromosomes from one parental line (human in hamster or mouse cells fused to human cells and mouse chromosomes in hamster–mouse hybrids). Parental lines are eliminated by selective growth conditions frequently involving gene complementation in the hybrid cells. Analysis of the DNA from these clones using Southern blot technology and knowledge of the karyotype of each

clone, makes it possible to unambiguously determine which chromosome contains the gene of interest. Although this technique is very time consuming to set up because many clones are required, once in place, it is a very rapid approach to determining the chromosome that contains a particular gene.

Although the procedure can be done rapidly under certain conditions, several problems are associated with this approach. First, somatic cell hybrids are typically unstable. Clones will usually contain a varying percentage of cells with slightly different karyotypes. In addition, gross (and submicroscopic) chromosomal alterations may be induced by this technique. This requires constant monitoring by isoenzyme analysis and can lead to ambiguous results. Second, unless specific deletions or translocations are present in the donor chromosomes, the resolution is limited to the chromosomal level. However, in many instances involving chromosomal abnormalities, this technique may help to provide definitive evidence that a particular gene is actually translocated. Finally, this technique is unsuitable for analyzing a large number of cell samples because of the extensive cell culturing involved. This procedure is therefore used mainly as a first-line technique for quickly determining the chromosome in which a particular cloned gene is situated.

C. *In Situ* Hybridization

The hybridization of nucleic acid probes directly to chromosomes provides the best resolution of gene localization short of detailed genetic analysis and nucleic acid sequencing. Developed in the late 1960s by Gall and Pardue (1969), this technique has been quite successful in localizing genes in polytene chromosomes and tandomly reiterated sequences in vertebrate species. Unfortunately, several attempts to localize unique sequences in human cells produced a wide range of artifacts attributed mainly to impure probes and resulted in skepticism about the usefulness of this technique. Within the last 5 years, procedures have gradually improved with the use of molecularly pure probes to the point where given a purified mRNA or a cloned DNA sequences, the chromosomal position of complementary sequences can be routinely determined in most species. One of the original approaches developed by Tereba *et al.* (1979) was to attach purified RNAs containing the sequences of interest to a heterologous double-stranded DNA which had been radioactively labeled with ^{125}I in such a manner as to retain a high molecular weight. The attachment was via hybrids between the poly(A) of the RNA and poly(BUdR) tails enzymatically attached to the heterologous DNA. Results of their studies were independently confirmed by chromosome fractionation studies and genetic analyses. The advantage of this approach was the relative quantitation available when more than one locus hybridized since the amount of radioactivity bound to any one locus was independent of the

size of the hybridizing sequence. The disadvantage included the requirement for highly purified RNA and an extensive preparation of the probe.

More recently, with the advent of cloned DNA, successful *in situ* hybridizations have been performed with nick-translated, cloned DNA by using either ^{3}H or ^{125}I. The hybridization conditions have also been improved with the use of dextran sulfate to increase the hybridization rate and 70% formamide at 70°C to denature the chromosomal DNA without destroying the details of the chromosomal banding patterns (Harper and Saunders, 1981). Even with these improvements, it is difficult to rationalize why the hybridization reactions work with these probes. The probes are usually short (although relatively large fragments are an important necessary factor) and not of sufficient specific activity to be detectable in single copies. One possible explanation is that many molecules are arrached at each site, thereby forming large networks of radioactive probe molecules. Regardless of the actual mechanism involved, these reactions have been very dependable when proper controls have been used and due care has been take) in aging the slides and with the rapid or low-temperature development of autoradiographs to limit background grains. Typically 5 to 30% of the grains over chromosomes can be attributed to specific hybridization in a successful experiment.

IV. *myc* and Its Association with B Cell Tumors

Of all the oncogene-related sequences, the chromosomal localization of c-*myc* has been examined most often and has generated the most interest. The prototype sequence is derived from the avian acute leukemia virus MC29. The virus is unusual in that it causes a wide range of diseases of both hematopoietic and solid tumor origin. The cell counterpart is highly conserved and can be detected in a wide variety of species (Shilo and Weinberg, 1981). Expression of the cellular gene has been observed in a variety of normal tissues at various stages of differentiation. It is thus likely that this gene plays a fundamental role in the growth or maintenance of cells. This gene is the first and prime example of a cellular gene's involvement in tumor formation. Neel *et al.* (1981), and others (Payne *et al.*, 1981, 1982; Hayward *et al.*, 1981), have shown that a large percentage of chicken bursal lymphomas induced by avian leukosis viruses are clonal and have the right-hand viral promoter region inserted near the cellular c-*myc* gene. High levels of c-*myc* mRNA containing viral sequences also were usually observed in these cells compared to 4-month normal bursal tissue (Hayward *et al.*, 1981). (It should be noted, though, that 5-day bursal tissue which contains actively growing B cells has elevated levels of c-*myc* mRNA also.) This discovery led to the promoter insertion model, which states that high levels of a

cellular protooncogene mRNA induced by the placement of an active transcriptional promoter next to the gene is responsible for the induction of oncogenesis (Neel *et al.*, 1981). In theory, any active promoter or enhancer sequence placed near a protooncogene should accomplish the same function as the viral promoter. Thus, transformation could, in practice, be accomplished by a chromosomal translocation. This theory is, in essence, at the heart of the rush to localize the protooncogenes.

A. Chromosomal Localization of c-*myc* on Chicken Chromosomes

The motivation for localizing the cellular c-*myc* gene in chickens was associated not with chromosomal translocations but rather with the desire to answer the question whether exogenous or endogenous retrovirus genomes were physically associated with protooncogenes. An understanding of this situation was important in ultimately determining the mechanism by which retroviruses occasionally incorporated protooncogenes into their genomes as well as determining the viral or cellular origin of this gene. The first approach was to partially separate chicken metaphase chromosomes from a fast-growing lymphoid cell line, MSB-1, by means of a sucrose gradient (Sheiness *et al.*, 1980). Due to the large size differential of the various chicken chromosomes, reasonable separation was accomplished between large, medium, and small chromosomes. Analysis of the extracted DNA from these fractionated chromosomes with a v-*myc* probe suggested that the c-*myc* sequences were located on a large chromosome.

In an independent set of experiments, Tereba and Lai (1982) analyzed chromosomes from normal chicken fibroblasts using *in situ* hybridization of RNA from MC29 virus, the prototype *myc*-containing virus, and MH2, a related virus also containing *myc* sequences, as well as cloned DNA containing the c-*myc* chicken sequences. All experiments showed hybridization over a large microchromosome probably between numbers 12 and 15. Exact determination was impossible because of the small size and uniform morphology of this group of chromosomes. Internal controls showing the location of *ev*1, an endogenous retrovirus locus, were consistent with previous studies (Tereba and Astrin, 1980). The inconsistency in these two sets of experiments are hard to rationalize, as other genes localized by these two methods have provided compatible results (i.e., *ev*1 and c-*src*). One explanation given has been the possibility that the *myc* gene was translocated in the MSB-1 cell line. A few alterations do exist in the karyotype of MSB-1, including an extra region on one of the number 1 chromosomes. However, unless MSB-1 cells only have one *myc* gene, this translocation theory would not easily explain the two divergent results. One approach that may resolve this discrepancy would be to perform *in situ* hybridization on chromosomes from MSB-1.

B. Mouse c-*myc* and Its Association with the t(12;15) Translocation of Plasmacytomas

Murine plasmacytomas have a frequently occurring specific translocation in which there is a reciprocal exchange between the distal part of chromosome 15 (15 D3/E) and chromosome 12 (12F2) or occasionally chromosome 6C2. In addition, trisomy of chromosome 15 is common in most T cell and some B cell leukemias. Since the immunoglobulin heavy chain gene had been localized to the breakpoint on chromosome 12 and the κ light chain gene was located on chromosome 6, this translocation event appeared very similar to the human Burkitt's lymphoma t(8;14) translocation to be described in Section IV,C and suggested some common mechanism of oncogenesis.

An examination of DNA clones containing the constant portion of the α immunoglobulin heavy chain gene from plasmacytomas revealed a rearrangement with nonimmunoglobulin DNA sequences being present near the α switch region. These sequences were called nonimmunoglobulin-associated rearranging DNA (NIARD) or lympohid rearranging DNA (LYR) (Harris *et al.*, 1982a; Adams *et al.*, 1982). The identity of these sequences was determined by hybridizing this cloned DNA with a v-*myc* probe (Adams *et al.*, 1983). As suggested by the chicken bursal lymphoma model system, the rearranging sequences did indeed contain the mouse c-*myc* gene. Examination of several plasmacytomas revealed that this rearrangement was not very specific at the molecular level with respect to the breakpoint on chromosome 12, that the c-*myc* gene had been decapitated of its 5′ intron in at least some cases leaving an altered gene, and that the union between the immunoglobulin gene and c-*myc* was in a 5′-to-5′ orientation.

As mouse chromosomes are very similar in morphology and form a continuous size gradient, *in situ* hybridization of these chromosomes is rarely attempted. Thus, the approach to confirm the chromosome position of c-*myc*—assumed to be on chromosome 15 from the molecular data described earlier and from the fact that the immunoglobulin heavy chain gene was previously localized to chromosome 12—relied on Chinese hamster–mouse somatic cell hybrids. Using cloned DNA probes containing NIARD sequences (shown to contain c-*myc* sequences), Harris *et al.* (1982b) and Calame *et al.* (1982) both showed a direct relationship between the presence of chromosome 15 and the NIARD sequences. Thus, by the combination of these data and the molecular analysis of the heavy chain gene in plasmacytomas, conclusive evidence is available that the c-*myc* gene is directly involved in the t(12;15) translocation.

Although the positions of the heavy chain immunoglobulin and the c-*myc* genes have been determined in the germline and have been shown to be rearranged in the t(12;15) translocation, the location of these genes in the translocated chromosomes remains to be determined. Either the heavy chain genes

could be transposed to chromosome 15 or c-*myc* to chromosome 12. From genetic and molecular considerations, Harris *et al.* (1982b) have proposed that the translocation is reciprocal with the breakpoint occurring at position 15D3 on chromosome 15, leaving all but the extreme 5′ terminus of c-*myc* on chromosome 15. The breakpoint on chromosome 12 would be at position 12F1, frequently in the C^{α} switch region. As a result of the orientation of the immunoglobulin genes, most of the constant region and all of the variable region would be retained on chromosome 12. The C^{α} region, however, would be translocated to chromosome 15. One consequence of this model would be the transposition of the c-*myc* promoter region and presumably regulator sequences to chromosome 12.

Unfortunately, as with the chicken c-*myc* gene, no direct evidence has been obtained concerning the oncogenic potential of the mouse c-*myc* gene involved in this translocation. The frequent relationship between translocation of this gene and plasmacytomas strongly implies that the gene is involved in some aspect of the oncogenic process. However, when plasmacytoma DNA is transfected into mouse NIH-3T3 cells, transformed cells are obtained which do not contain the rearranged c-*myc* gene (Lane *et al.*, 1982). In addition, levels of expression of c-*myc* may not be altered in all plasmacytomas as compared to normal B cells (Shen-Ong *et al.*, 1982), although other groups have noticed such changes (Muskinski *et al.*, 1983; Marcu *et al.*, 1983). Thus, a two-step process must be envisioned if c-*myc* is to be part of the oncogenic mechanism.

C. Involvement of Human c-*myc* in the t(8;14) Translocation of Burkitt's Lymphoma

With the discovery that c-*myc* was involved in chicken lymphomas via a viral integration mechanism, there was a concerted effort to show a linkage between human c-*myc* and Burkitt's lymphoma. As mentioned in the introduction to Section IV, translocations fit into the general hyp sis of the promoter insertion theory. Previous karyotology on chromosomes obtained from Burkitt's lymphoma revealed a consistent reciprocal translocation between chromosomes 8 at band q24 and chromosome 14 at band q32. Occasional variations in the translocation were also observed between chromosome 8q24 and 2p12 or 22q11 (Sandberg, 1980). Previous studies, including *in situ* hybridization studies, revealed that the immunoglobulin heavy chain gene family was located on chromosome 14q32 (Croce *et al.*, 1979; Kirsch *et al.*, 1982) and that the light chain κ and λ genes were located on chromosomes 2 and 22, respectively (Erikson *et al.*, 1981; McBride *et al.*, 1982a; Malcolm *et al.*, 1982). It was therefore theorized that c-*myc* would be found on chromosome 8.

Several groups independently showed that this postulated position was indeed