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Integration of transfected LTR sequences into the c-*raf* proto-oncogene: activation by promoter insertion

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A malignant cell line (clone S1) isolated after co-transfection of normal NIH3T3 DNA and Moloney leukemia virus long terminal repeat (Mo-LTR) sequences has previously been described to contain an activated c-*raf* oncogene. Here, we report the isolation by molecular cloning and the structural analysis of the LTR-activated c-*raf* gene. As shown by Southern blot and nucleotide sequence analyses, the transfected Mo-LTR sequences integrated into the 5th intron of the endogenous c-*raf* proto-oncogene. This intragenic LTR insertion led to the expression of high levels of LTR-U5-c-*raf* hybrid transcripts indicating an initiation of transcription from the Mo-LTR promoter. Transcriptional activation of c-*raf* is accompanied by the synthesis of large amounts of cytoplasmic c-*raf* protein. Immunoblot analysis suggests that the proteins encoded by the LTR-activated c-*raf* gene are truncated compared with the normal c-*raf* gene product(s). Our results indicate a promoter insertion mechanism of c-*raf* activation.

Key words: c-*raf* oncogene/activation/long terminal repeat/promoter insertion

Introduction

In an attempt to establish a novel strategy for the identification of potential cellular oncogenes (c-*onc* genes) malignant cell lines were isolated after co-transfection of normal NIH3T3 (carrier) DNA and cloned Moloney leukemia virus long terminal repeat (Mo-LTR) sequences onto NIH3T3 recipient cells (Müller and Müller, 1984). Theoretically, such an approach can lead to the induction of neoplastic transformation in several ways, including (i) the transcriptional activation of neighbouring genes, *via* the Mo-LTR promoter or enhancer, (ii) block of gene transcription as a consequence of Mo-LTR integration, (iii) transcriptional activation of carrier DNA sequences by juxtaposition to cellular enhancers or promoters [as suggested by Cooper *et al.* (1980)], or (iv) expression of structurally aberrant proteins from truncated, rearranged or mutated carrier DNA sequences. While several of the neoplastic cell lines obtained by this approach apparently contain activated oncogenes that are different from 21 previously described c-*onc* genes, the transforming gene of one of these clones (designed clone S1; Müller and Müller, 1984) was shown to be homologous to the murine retroviral oncogene v-*raf* (Rapp *et al.*, 1983), which in turn is homologous to one of the two oncogenes (v-*mil*) transduced by the avian leukemia virus MH2 (Sutrave *et al.*, 1984). The *raf/mil* oncogene has thus been identified in three independent ways.

Analysis of DNA from 15 secondary (2°) and tertiary (3°) foci derived from clone S1 revealed a single Mo-LTR integration site located on a specific restriction fragment, indicating a linkage between the activated oncogene and Mo-LTR sequences. To clarify the mechanism of oncogene activation in clone S1 we have isolated, by molecular cloning, the Mo-LTR-c-*raf* complex and analyzed its structure and expression. The results described here show that the oncogenic activation of c-*raf* occurred by Mo-LTR integration into the 5th intron of the proto-oncogene leading to the synthesis of high levels of c-*raf* transcripts starting in the Mo-LTR. Transcriptional activation of c-*raf* is accompanied by the synthesis of large amounts of cytoplasmic c-*raf* protein. These findings indicate a promoter insertion mechanism of c-*raf* activation.

Results

Transcriptional activation of c-raf: transcripts initiate in Mo-LTR region

It has previously been shown by dot blot analysis that 2° and 3° foci derived from clone S1 express high levels of Mo-LTR-U5 sequences, whereas expression of the Mo-LTR-U3 region was not detectable (Müller and Müller, 1984). As displayed in

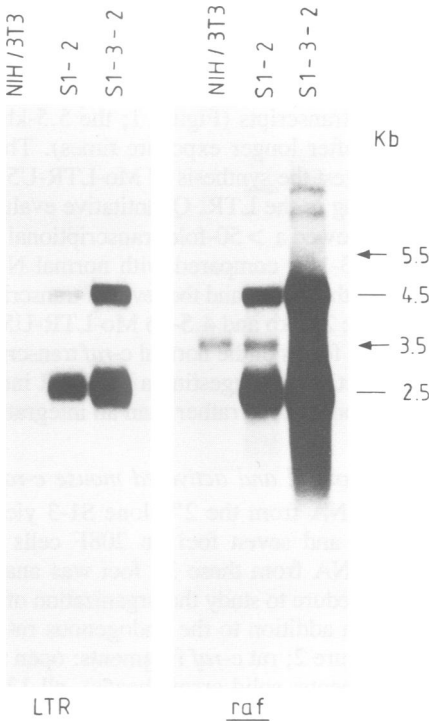

Fig. 1. Analysis of Mo-LTR and c-*raf* RNA expression in normal NIH3T3 cells and 2° (S1-2) and 3° (S1-3-2) clones containing activated c-*raf* genes. Endogenous c-*raf* mRNAs are indicated by arrows. 5 μg of polyadenylated RNA were applied to each lane, separated in formaldehyde-agarose gels, blotted onto nitrocellulose paper and hybridized to Mo-LTR (*Pvu*II-*Pst*I fragment carrying part of U3 and all U5 sequences; Müller and Müller, 1984) and v-*raf*- (*Xho*I-*Sst*II fragment; Rapp *et al.*, 1983) specific probes. 18S and 28S rRNAs were used as size markers.

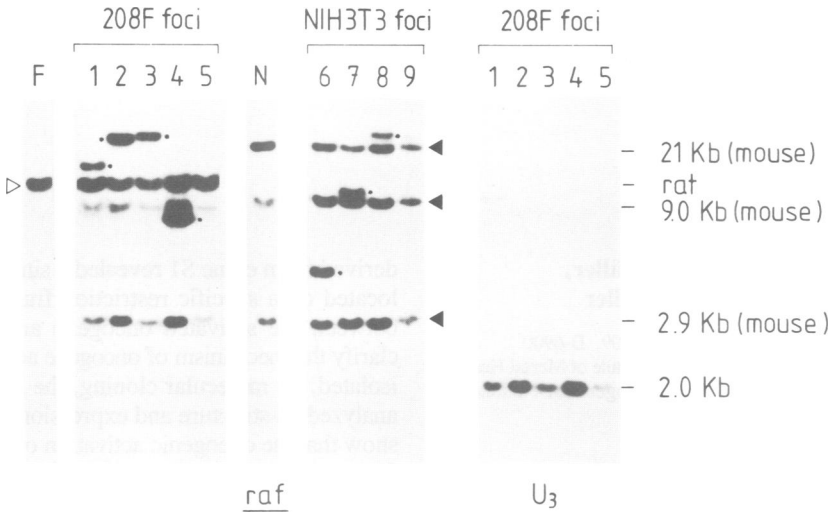

Fig. 2. Southern blot analysis of *Eco*RI-digested genomic DNA from normal 208F **(lane F)** and NIH3T3 **(lane N)** cells, from various 3° 208F-derived **(lanes 1–5)** and NIH3T3-derived **(lanes 6–8)** foci, and from the 1° focus S1 **(lane 9)**. Endogenous rat c-*raf* fragments are indicated by open arrow heads, c-*raf* bands identical in size to endogenous mouse c-*raf* fragments are marked by solid arrow heads. Bands that are different from normal mouse or rat c-*raf* fragments are marked by dots. Hybridization to v-*raf* or Mo-LTR-U3 sequences was performed as described (Müller and Müller, 1984). **Lanes 1–5:** clones F/S1-3-1, F/S1-3-2, F/S1-3-3, F/S1-3-4, F/S1-3-5; **lanes 6–8:** clones S1-3-1, S1-3-2 and S1-3-3. Mobility of *Hind*III-digested λDNA is indicated.

Figure 1, two major polyadenylated RNAs of ~2.5 kb and 4.5 kb containing Mo-LTR-U5 sequences could be identified in clone S1-2 and S1-3-2 cells in addition to several larger RNAs that were present in much lower quantities. The 4.5-kb and the larger RNA molecules presumably represent immature transcripts (J.Cleveland and U.Rapp, unpublished observation). When the same clones were analyzed for c-*raf* expression (Figure 1), two major RNAs of very similar size to the Mo-LTR-U5 transcripts of 2.5 kb and 4.5 kb could be identified in addition to two minor transcripts of 3.5 and 5.5 kb. The latter RNAs, however, were also detected in normal NIH3T3 cells and, therefore, represent endogenous c-*raf* transcripts (Figure 1; the 5.5-kb mRNA can only be detected after longer exposure times). These observations strongly suggest the synthesis of Mo-LTR-U5-c-*raf* hybrid transcripts initiating in the LTR. Quantitative evaluation of blot autoradiograms showed a >50-fold transcriptional activation of c-*raf* in clone S1-3-1 as compared with normal NIH3T3 cells. As judged from both the size and the level of transcripts it appears to be likely that the 2.5-kb and 4.5-kb Mo-LTR-U5-c-*raf* RNAs represent truncated forms of the normal c-*raf* transcripts of 3.5 kb and 5.5 kb, respectively, suggesting a Mo-LTR integration into a 5′-terminal region of c-*raf* rather than an integration upstream from the gene.

Organization of normal and activated mouse c-raf genes

Transfection of DNA from the 2° clone S1-3 yielded six foci on NIH3T3 cells and seven foci on 208F cells (Müller and Müller, 1984). DNA from these 3° foci was analyzed by the Southern blot procedure to study the organization of the activated c-*raf* oncogene. In addition to the endogenous rat or mouse c-*raf* sequences (Figure 2; rat c-*raf* fragments: open arrow heads; mouse c-*raf* fragments: solid arrow heads), all 13 clones were found to contain additional DNA fragments hybridizing to the v-*raf*-specific probe (Figure 2; lanes 1–5 and 6–8; and unpublished observations). *Eco*RI digestion of normal NIH3T3 DNA yielded three fragments of 21 kb, 9 kb and 2.9 kb (Figure 2; lane N). The two smaller fragments were found in all 3° 208F foci, whereas the larger fragment of 21 kb could not be detected in any of these clones (Figure 2; lanes 1–5). Four clones, however, contained a third fragment of variable size (~8–25 kb; Figure 2; lanes 1–4; marked by dots). Similarly, additional c-*raf* fragments of variable size were also found in all 3° NIH3T3 clones (Figure 2; lanes 6–8; marked by dots).

In one 3° 208F clone (F/S1-3-5; Figure 2, lane 5), only two

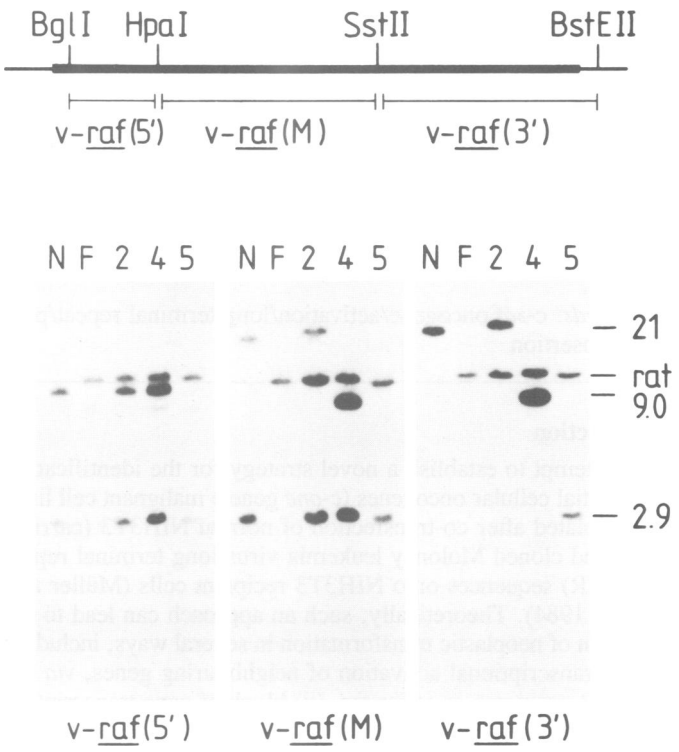

Fig. 3. Southern blot analysis of normal and activated c-*raf* genes using probes specific for the 5′ terminus of v-*raf* [*Bgl*I-*Hpa*I fragment; v-*raf* (5′)], the middle region of v-*raf* [*Hpa*I-*Sst*II fragment; v-*raf*(M)], and the 3′ terminus of v-*raf* [*Sst*II-*Bst*EII fragment; v-*raf*(3′)] (see top panel). Bold lines, v-*raf* sequences; thin lines, helper viral sequences. **Lane N:** normal NIH3T3 DNA; **lane F:** normal 208F DNA; **lanes 2,4,5:** 3° 208F-derived clones F/S1-3-2, F/S1-3-4 and F/S1-3-5. Sizes of normal mouse c-*raf* fragments (21 kb, 3′ terminus; 9.0 kb, 5′ terminus and 2.9 kb, middle region) and of the endogenous 208F rat homolog are indicated.

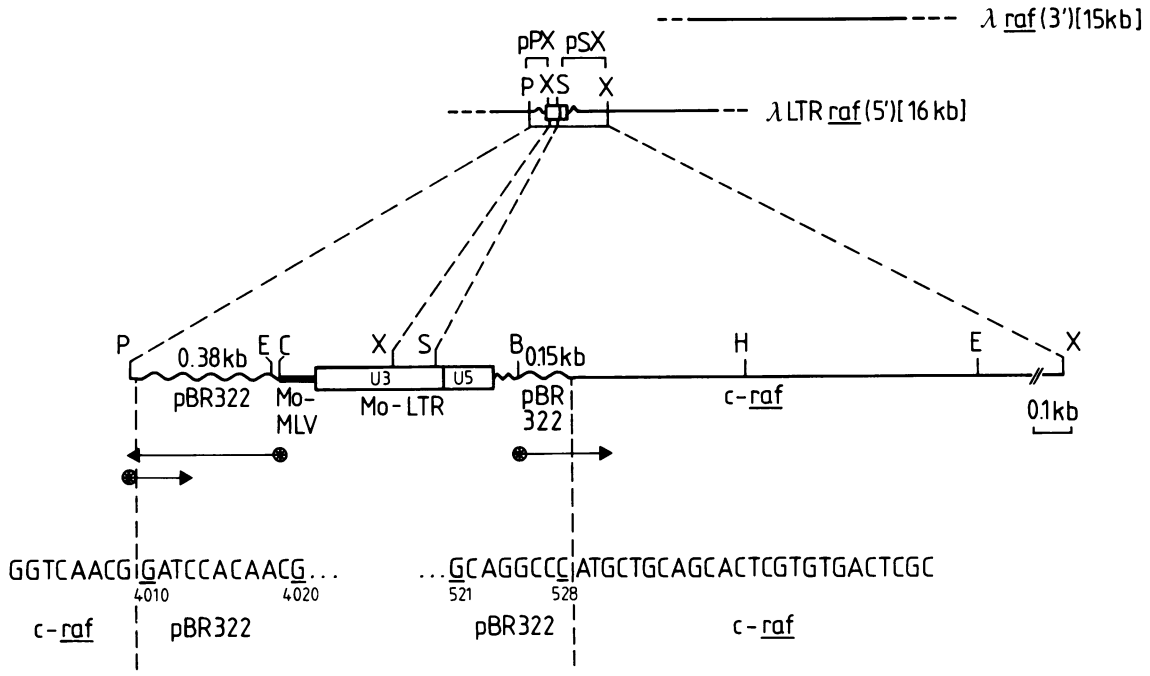

Fig. 4. Structure of recombinant EMBL3 phage clones containing Mo-LTR and 5′-c-*raf* sequences [λLTR*raf*(5′)] or 3′-c-*raf* sequences [λ*raf*(3′)]. Termini of phage inserts have not been precisely mapped, as indicated by the dotted lines. Two plasmids, pPX and pSX, containing the indicated regions of λLTR*raf*(5′) were used for nucleotide sequence analysis. Wavy lines, pBR322 sequences of 3′-flanking cellular sequences from pLTR3 used in the original transfection (see Müller and Müller, 1984); bold lines, Mo-MLV non-LTR sequences; boxes, Mo-LTR-U3 and -U5 sequences; thin lines, c-*raf* sequences. Arrows indicate start and direction of sequence analyses. ^{32}P-end-labeling of restriction fragments was carried out using either *E. coli* DNA polymerase I or polynucleotide kinase. Nucleotide sequences of pLTR3/c-*raf* junctions are shown at the bottom. B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sac*I; X, *Xba*I.

mouse c-*raf* fragments were detectable. As this could be taken as an indication that part of the c-*raf* gene may be missing in this clone and would thus be dispensable for transformation, it was important to map the three *Eco*RI fragments of the mouse c-*raf* gene with regard to their location within the gene. For this purpose probes were delineated that were specific for the 5′ terminus (*Xho*I-*Hpa*I), the middle region (*Hpa*-*Sac*II) and the 3′ terminus (*Sac*II-*Bst*EII) of v-*raf* (Figure 3; Rapp *et al.*, 1983). Using these probes it was possible to assign the 21-kb *Eco*RI fragment to the 3′ terminus of c-*raf*, the 2.9-kb fragment to the middle portion and the 9-kb fragment to the 5′ region of the mouse c-*raf* gene (Figure 3). The data shown in Figures 2 and 3 can now be conclusively interpreted: both the 5′-terminal 9-kb c-*raf* fragment and the 2.9-kb middle fragment of c-*raf* are entirely contained in the Mo-LTR activated c-*raf* gene, whereas the 21-kb 3′-terminal fragment was truncated to variable degrees in each clone (Figure 3; lanes 1 – 5). Even the smallest 3′ fragment of ~3 kb (which co-migrates with the 2.9-kb middle fragment in Figure 2; lane 5) is still big enough to carry the entire 3′ terminus of the normal c-*raf* gene (M.Goldsborough and U.Rapp, unpublished data). It can thus be concluded that the activated c-*raf* genes in all foci analyzed harbor the entire information contained in v-*raf* as well as the normal 3′-non-coding sequences of c-*raf* including the polyadenylation site. In the Southern blot analysis of DNA from the 1° clone S1, no bands other than the endogenous c-*raf* bands are visible (Figure 2; lane 9). This finding would be compatible with the conclusion that the transfected Mo-LTR sequences were inserted into the endogenous c-*raf* gene rather than having been linked to transfected carrier DNA sequences.

Amplification of c-raf and Mo-LTR sequences in tertiary foci

As shown by Southern blot analysis, both exogenous c-*raf* sequences and Mo-LTR sequences are present as multiple copies

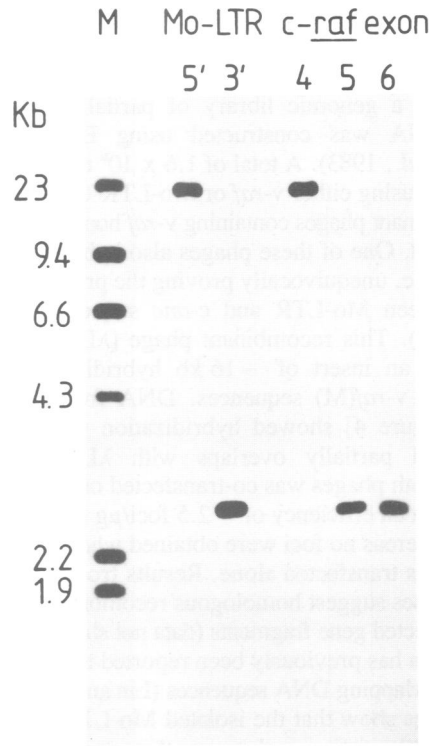

Fig. 5. Hybrdization of λLTR*raf*(5′) *Sac*I fragments with Mo-LTR- and exon-specific ^{32}P-Labeled probes. Mo-LTR-5′ (U3) probe: *Cla*I-*Sac*I fragment; Mo-LTR-3′ (mostly U5): *Sac*I-*Bam*HI probe (see Müller and Müller, 1984 and Figure 4); c-*raf* exon 4 probe: 2.6-kb *Eco*RI-*Sph*I fragment from plasmid p755 derived from a human genomic c-*raf* clone (T.Bonner *et al.*, in preparation); exon 5 probe: 1.6-kb *Eco*RI-*Sph*I fragment from plasmid p755; exon 6 probe: 0.3-kb *Eco*RI-*Sal*I fragment from plasmid p359 derived from a human *c-raf* clone. M,^{32}P-labeled *Hind*III-digested λDNA.

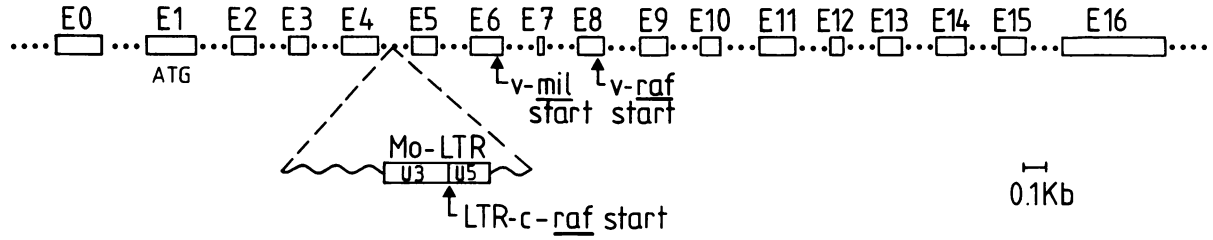

Fig. 6. Schematic representation of the Mo-LTR activated c-*raf* gene, based on the structure of the human homolog (T.Bonner *et al.*, in preparation). Boxes represent exons, dotted lines show the positions of intervening or non-translated terminal sequences. Start of v-*mil* and v-*raf* genes is indicated. Exon 0 and part of exon 1 of c-*raf* are non-coding as indicated by nucleotide sequence analysis (M.Goldsborough and U.Rapp, unpublished data). The presumptive initiation codon (ATG) of normal c-*raf* protein is in exon 1.

in the genome of several 3° foci (e.g., F/S1-3-2, F/S1-3-4; Figures 2 and 3; lanes 2 and 4) indicating an amplification of the Mo-LTR/c-*raf* complex. This amplification involved all three c-*raf* *Eco*RI fragments and thus apparently the entire activated gene (Figure 2). The greatest amplification was found in clone F/S1-3-4 which contained at least 10 copies of both Mo-LTR and mouse c-*raf* (Figure 2). Amplification of the LTR-activated c-*raf* gene was also detected in several 3° NIH3T3 derivatives of clone S1 (data not shown). In all clones analyzed, gene amplification resulted in an increased expression of U5-c-*raf* RNA (compare for instance clone S1-2 containing a single copy of the activated c-*raf* gene with clone S1-3-2 which carries more than five copies; Figure 1). Amplification of oncogenes transferred in 2° or 3° transfections has been found to occur also with other oncogenes (R.Müller and D.Müller, unpublished observation), but the mechanism of such amplification events remains obscure.

Molecular cloning of LTR-activated c-raf gene

To be able to determine the precise Mo-LTR integration site, the activated c-*raf* gene was isolated by molecular cloning. For this purpose, a genomic library of partially *Sau*3A-digested F/S1-3-4 DNA was constructed using EMBL3 as vector (Frischauf *et al.*, 1983). A total of 1.6 x 10^6 recombinant phages was screened using either v-*raf* or Mo-LTR-U3-specific probes. Eight recombinant phages containing v-*raf* homologous sequences were obtained. One of these phages also hybridized to the Mo-LTR-U3 probe, unequivocally proving the previously suggested linkage between Mo-LTR and c-*onc* sequences (Müller and Müller, 1984). This recombinant phage [λLTR*raf*(5′); Figure 4] contained an insert of ~16 kb hybridizing to Mo-LTR, v-*raf*(5′) and v-*raf*(M) sequences. DNA from another phage [λ*raf*(3′); Figure 4] showed hybridization with v-*raf*(M) and v-*raf*(3′) and partially overlaps with λLTR*raf*(5′). When DNA from both phages was co-transfected on 208F cells, focus induction with an efficiency of ~2.5 foci/μg DNA/10^6 cells was observed, whereas no foci were obtained when either of the two fragments was transfected alone. Results from nucleic acid and protein analyses suggest homologous recombination between the two co-transfected gene fragments (data not shown). Homologous recombination has previously been reported to occur after transfection of overlapping DNA sequences (Lin and Sternberg, 1984). These findings show that the isolated Mo-LTR-c-*raf* sequences and biologically active and prove that c-*raf* is the activated oncogene of clone S1.

Nucleotide sequence analysis of Mo-LTR/c-raf junctions

For nucleotide sequence analysis, a *Pst*I-*Xba*I fragment of λLTR*raf*(5′) carrying the Mo-LTR-U3 and 5′-flanking sequences and a *Sac*I-*Xba*I fragment containing Mo-LTR-U5 and 3′-flanking sequences were subcloned in pUC18 (Norrander *et al.*, 1983) (Figure 4; recombinant plasmids pPX and pSX, respectively). Both plasmids were cleaved at the indicated positions and end-labeled as described in the legend to Figure 4. Nucleotide sequence analysis was performed according to the method of Maxam and Gilbert (1980). The sequences of 5′ and 3′ junctions of c-*raf* and pBR322 (flanking the transfected pLTR3 sequences; see also Figure 2 , last lane) are displayed in Figure 4 (bottom). However, when these 'flanking' mouse c-*raf* sequences were compared with the nucleotide sequence of human c-*raf* cDNA, no homology was detectable. Since mouse and human c-*raf* exons are highly homologous and Mo-LTR sequences appear to be located within the c-*raf* gene (Figure 1), this finding suggests that integration of the transfected Mo-LTR occurred in an intron of the mouse c-*raf* gene.

Mo-LTR integration site in the 5th intron of c-raf

To verify the hypothesis that the transfected Mo-LTR is located within the c-*raf* gene, sequences located either 5′or 3′ to the LTR in λLTR*raf*(5′) DNA were analyzed for hybridization to exon-specific probes derived from the human c-*raf* gene. Figure 5 shows that a *Sac*I fragment of ~20 kb hybridized to both the 5′ region of Mo-LTR and to exon 4 of c-*raf*, but not to exons 5 and 6. In contrast, another *Sac*I fragment of 2.8 kb hybridized to both the Mo-LTR 3′ region and to exons 5 and 6 of c-*raf*, but not to exon 4. These results demonstrate that the transfected Mo-LTR is located between exon 4 and exon 5, i.e., in the 5th intron of the c-*raf* gene. A schematic representation of the organization of the activated c-*raf* gene is shown in Figure 6, based on the structure of the human c-*raf* gene which is very similar to the mouse homolog with respect to exon structure (M.Goldsborough and U.Rapp, unpublished data). These data lend strong support to our previous conclusion (see above and Figure 2; lane 9) that in the 1° clone S1 the transfected Mo-LTR recombined with the endogenous c-*raf* gene of the recipient cell.

Increased expression of cytoplasmic c-raf protein in clone S1 cells

To investigate whether the transcriptional activation of c-*raf* by the transfected Mo-LTR resulted in an increased synthesis of c-*raf*, protein immunofluorescence analyses using *raf*-specific antibodies (A.Schultz, H.Oppermann and U.Rapp, in preparation) were carried out. As shown in Figure 7, strong cytoplasmic fluorescence was detectable in clone S1-3-4 cells using antisera directed against either a peptide derived from the middle region of v-*raf* (αSP46; Figure 7A), a peptide specific for the carboxy terminus of v-*raf* (αSP63; Figure 7D) or a *raf* protein synthesized in *Escherichia coli* (α-*raf*; Figure 7G). The observed cytoplasmic fluorescence appears to be specific for the c-*raf* gene product, as (i) no staining was found with 208F cells (Figure 7A,D,G), (ii) staining of S1-3-4 cells by anti-peptide sera could be blocked by pre-incubation of the antibodies with the respective peptides (Figure 7C,F), and (iii) clone S1-3-5 cells (containing a single copy of the Mo-LTR-c-*raf* complex) showed a

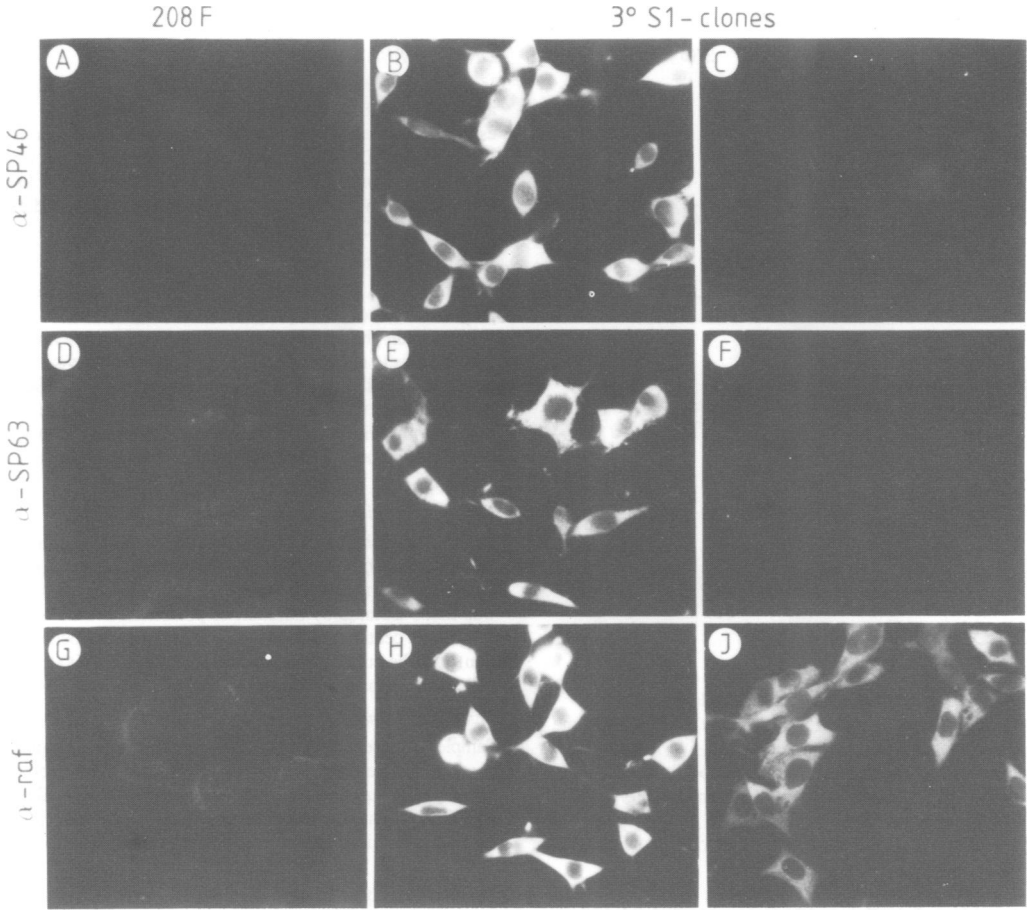

Fig. 7. Immunofluorescence analysis of c-*raf* protein expression in normal 208F cells **(A,D,G)**, F/S1-3-4 cells **(B,C,E,F,H)** and in F/S1-3-5 cells **(J)**. Antibodies used were either raised against synthetic peptides derived from the v-*raf* middle region (αSP46; **A,B,C**) or the v-*raf* 3′ terminus (αSP63; **D,E,F**) or directed against v-*raf* protein synthesized in *E. coli* **(G,H,J)** (A.Schultz, H.Oppermann and U.Rapp, in preparation). **C** and **F**, same as **B** and **E**, respectively, but antiserum was pre-incubated with synthetic peptide (SP46 in **C**; SP63 in **F**). Fixation of cells in Sainte-Marie reagent and processing of slides was as described (Müller and Wagner, 1984). Rhodamine-conjugated goat anti-rabbit IgG was used as the second antibody. Microscopic magnification, 400x.

weaker fluorescence than S1-3-4 cells (carrying >10 copies of the activated c-*raf* gene) (Figure 7J). Data obtained by immunoblot analysis in conjunction with cell fractionation indicate the synthesis of multiple forms of cytoplasmic c-*raf* protein in the range of 44 – 50 kd in F/S1-3-4 cells, whereas in normal 208F cells only low levels of *raf*-reactive protein in the range of 75 kd were detectable under the same experimental conditions (Figure 8). The mol. wt. of the latter protein, however, is very close to the coding capacity of the normal c-*raf* protein (648 amino acids = 74 kd; deduced from the nucleotide sequence of human c-*raf* cDNA) (T. Bonner *et al.*, in preparation). It is thus possible that the band of ~75 kd observed in normal 208F corresponds to an endogenous c-*raf* gene product. This would suggest that the products encoded by the LTR-activated c-*raf* gene are truncated compared with normal c-*raf* protein, a conclusion that is compatible with the intragenic integration site of the Mo-LTR. However, further studies (e.g., peptide mapping and pulse-chase experiments) will have to clarify the exact nature of the 75 kd band as well as the origin of other minor *raf*-reactive protein species observed in other cellular compartments (Figure 8).

Discussion

In a previous paper we reported the isolation of malignant cell lines after co-transfection of normal NIH3T3 DNA and Mo-LTR sequences (Müller and Müller, 1984). In one of these clones (S1), the activated oncogene was identified as c-*raf* which appeared to be linked to Mo-LTR sequences located on a specific restriction fragment. The present study was undertaken to determine the precise integration site of the transfected Mo-LTR and to elucidate the mechanism of c-*raf* activation.

The retroviral LTR contains sequence structures required for viral integration into the host cell genome, for initiation and termination of transcription and for polyadenylation of viral mRNA (Temin, 1982). There are at least two different elements in the LTR that can positively influence transcriptional activity of contiguous sequences: (i) a presumptive promoter ('TATA box') near the 3′ end of the U3 sequence (Van Beveren *et al.*, 1980; Yamamoto *et al.*, 1980; Fuhrmann *et al.*, 1981), and (ii) an enhancer sequence residing in that region of U3 where tandem repeats are found (Levinson *et al.*, 1982; Jolly *et al.*, 1983; Luciw *et al.*, 1983). Transcriptional activation of adjacent DNA sequences by the first mechanism, i.e., initiation of transcription in the LTR ('down-stream promotion'), requires LTR insertion upstream from these sequences and integration in the same transcriptional orientation (Payne *et al.*, 1982). In contrast, activation of transcription by the U3 enhancer sequence occurs with similar efficiencies when the LTR becomes integrated into the flanking sequences on either side of the gene regardless of the orientation (Payne *et al.*, 1982; Jolly *et al.*, 1983; Luciw *et al.*, 1983).

As shown in Figure 1, the analyzed 2° and 3° foci derived from clone S1 express high levels of 2.5-kb and 4.5-kb transcripts

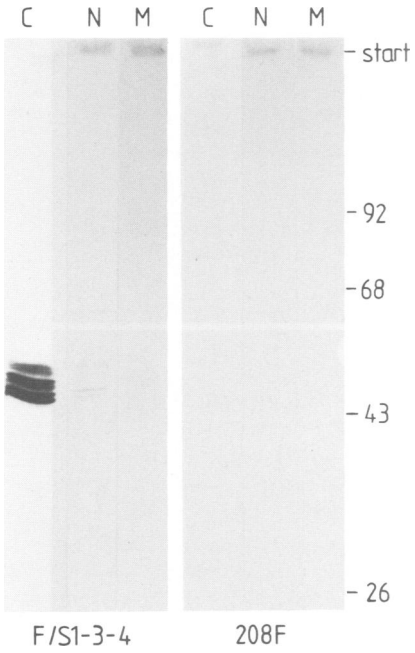

Fig. 8. Immunoblot analysis of c-*raf* proteins from the 3° clone F/S1-3-4 and from 208F cells following cell fractionation. C, cytoplasmic fraction (100 000 *g* supernatant); N, nuclear fraction (5000 *g* pellet); M, microsome fraction (100 000 *g* pellet). Proteins were separated on 10–15% gradient polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes, incubated with αSP63 antiserum and subsquently with peroxidase-labeled anti-rabbit IgG and finally reacted with chloronaphthol as a peroxidase substrate. Numbers indicate migration of marker proteins (UD).

containing both Mo-LTR-U5 and c-*raf* sequences, but no U3 sequences. These transcripts, however, are truncated compared with the normal c-*raf* RNAs (Figure 1). These findings suggest that (i) transcription of the activated c-*raf* gene initiates from the presumptive promoter ('TATA box') of the LTR; (ii) Mo-LTR sequences integrated upstream from v-*raf*-homologous sequences, and (iii) the Mo-LTR-U5-c-*raf* transcripts have shorter 5′-terminal sequences than the normal c-*raf* transcripts. These conclusions are fully supported by structural analyses of the molecularly cloned LTR-activated c-*raf* gene, which show that the Mo-LTR integration site is located in the 5th intron of the proto-oncogene. In addition, immunoblot analyses suggest the synthesis of truncated c-*raf* gene products in F/S1-3-4 cells (Figure 8). Interestingly, both retroviral oncogenes v-*mil* and v-*raf* also lack sequences homologous to several amino-terminal coding exons of c-*raf* (five in v-*mil*; seven in v-*raf*; Figure 6). It thus cannot be excluded that such structural alterations play a role in the activation of the oncogenic potential of c-*raf*. Although our results clearly show the synthesis of large amounts of c-*raf* protein (Figures 7,8), the precise initiation of translation remains unknown. The 5th coding exon of c-*raf*, however, contains multiple potential ATG initiation codons (T.Bonner *et al.*, in preparation). We have started to purify by affinity chromatography and h.p.l.c. c-*raf* protein from F/S1-3-4 cells to be able to determine the amino-terminal sequence of the protein.

Our results clearly show an LTR-mediated promoter insertion mechanism of c-*raf* activation. This observation indicates that the induction of neoplastic transformation by transfection of retroviral LTR sequences onto NIH3T3 cells is possible – albeit with low efficiency. It will now be intriguing to investigate the nature of the other (unknown) c-*onc* genes detected by the same approach (Müller and Müller, 1984) and to unravel the mechanism of their activation.

Materials and methods

DNA and RNA analyses

Isolation and restriction analyses of DNA were carried out as described (Müller and Müller, 1984). Polyadenylated RNA was isolated, separated by electrophoresis in formaldehyde-agarose gels and blotted as previously reported (Müller *et al.*, 1984). All DNA fragments used for nick-translation were purified from vector sequences by preparative agarose gel electrophoresis. In all experiments, blots were washed in 0.2 x SSC at 68°C for 3 x 30 min and exposed to Kodak XAR films at −70°C for 3–6 h.

Construction and screening of genomic libraries

Genomic DNA was partially digested with *Sau*3A and cloned into the *Bam*HI polylinker site of the λ phage-derived EMBL3 vector (Frischauf *et al.*, 1983). Recombinant phages were propagated on a selective host (NM539) and screened after transfer to nitrocellulose filters (Benton and Davis, 1977) without prior amplification of the library using v-*raf* or Mo-LTR-U3 specific, nick-translated probes. After washing the filters under stringent conditions (0.2 x SSC at 68°C for 3 x 30 min) hybridization of the Mo-LTR-U3 specific probe to phages containing endogenous mouse or rat LTR sequences was not detectable (Müller and Müller, 1984).

Cell fractionation

Cells were washed with phosphate-buffered saline (PBS) at 0°C, swollen in three volumes of hypotonic buffer (20 mM triethanolamine; 5 mM $MgCl_2$, 1 mM dithiothreitol) for 15 min at 0°C and broken in a Dounce homogenizer. The homogenate was made isotonic by addition of potassium acetate to a final concentration of 0.1 M and centrifuged for 10 min at 5000 *g*. The pellet was saved (nuclear fraction) and the supernatant was spun again at 100 000 *g* and then separated into pellet (microsome fraction) and supernatant (cytoplasmic fraction).

Immunoblot analysis

Samples were separated on 10/15% gradient polyacrylamide gels (Laemmli, 1970). Proteins were electrophoretically transferred to nitrocellulose membranes, incubated with c-*raf*-specific antibodies (αSP63; 1:100 diluted in 10% calf serum) and finally treated with peroxidase-labeled goat anti-rabbit IgG (Towbin *et al.*, 1979). As a substrate for peroxidase reaction chloronaphthol was used.

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