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Mutations around the NG59 lesion indicate an active association of polyoma virus middle-T antigen with pp60$^{c\text{-}src}$ is required for cell transformation

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The transforming activity of polyoma virus middle-T antigen is believed to be dependent on its ability to form a complex with the cellular tyrosine protein kinase, pp60$^{c\text{-}src}$. This hypothesis is based on observations of mutants of middle-T which demonstrated a correlation between these two activities. To investigate further the significance of pp60$^{c\text{-}src}$ association in transformation by middle-T, a series of deletion and point mutants were constructed around the NG59 lesion since this region has been implicated in pp60$^{c\text{-}src}$ binding. Analysis of the middle-T variants revealed a complete correlation between the presence of associated activated pp60$^{c\text{-}src}$ and the ability to transform. Further, this ability of pp60$^{c\text{-}src}$ to associate with middle-T may depend on the presence of a β-turn between amino acids 177 and 180. The results indicate the NG59 phenotype results from the introduction of an isoleucine residue between amino acids 177 and 178 rather than the transition mutation at 179. The mutant MG1 is a single point mutation (at residue 180) and represents the smallest change in the middle-T which abolishes both the transforming and kinase activity of middle-T. Taken together, the data suggest the region surrounding the NG59 lesion is involved in the formation of an active complex between middle-T and pp60$^{c\text{-}src}$ and strongly suggest that this association is an absolute requirement for polyoma virus-induced transformation.

Key words: middle-T/mutants/pp60$^{c\text{-}src}$/protein tyrosine kinase/transformation

Introduction

The middle-T antigen encoded by the early region of the polyoma virus genome alone is sufficient to initiate and maintain neoplastic transformation of established rodent cell lines (Triesman *et al.*, 1981). The biochemical basis underlying this property of middle-T, however, remains largely unknown (for review, see Tooze, 1981; Smith and Ely, 1983). The membrane-associated middle-T antigen has been shown to possess an associated tyrosine kinase activity in immunoprecipitates (Eckhart *et al.*, 1979; Schaffhausen and Benjamin, 1979; Smith *et al.*, 1979), and the presence of the kinase activity correlates with the ability of different mutants to transform.

Recently, evidence has been presented which suggests that the kinase activity is not an intrinsic property of the molecule itself but rather results from the formation of a stable complex between the middle-T antigen and pp60$^{c\text{-}src}$ (Courtneidge and Smith, 1983, 1984). One consequence of the interaction between transformation-competent middle-T antigens and pp60$^{c\text{-}src}$ appears to be the stimulation of the specific activity of the pp60$^{c\text{-}src}$ tyrosine kinase (Bolen *et al.*, 1984; Courtneidge, 1985). Since there are no known transformation-competent mutants of polyoma which lack middle-T antigen-associated kinase activity and as tyrosine-specific kinases have been implicated in transformation by retroviruses, it has been suggested that transformation by polyoma virus somehow involves the tyrosine kinase activity of pp60$^{c\text{-}src}$. Further, the finding that the fraction of pp60$^{c\text{-}src}$ bound to middle-T is present in an altered phosphorylated state (Courtneidge, 1985; Yonemoto *et al.*, 1985) has led to the suggestion that middle-T transforms by disrupting the mechanisms which normally modulate the kinase activity of pp60$^{c\text{-}src}$.

Hence a current hypothesis for transformation by polyoma virus middle-T requires that it forms an active complex with pp60$^{c\text{-}src}$. Although an analysis of mutants of middle-T antigen revealed a correlation between the ability to transform and exhibit *in vitro* kinase activity, there is insufficient evidence to indicate that pp60$^{c\text{-}src}$ association is an absolute requirement for transformation by middle-T. This is illustrated by the fact that there are only a few mutants described to date (e.g. NG59, SD15 and 1387T) which exhibit a transformation-defective and kinase-inactive phenotype (Carmichael *et al.*, 1982; Courtneidge and Smith, 1984). The mutant middle-T antigen encoded by SD15 encompasses a large deletion of 47 amino acids and five point mutations (Carmichael and Benjamin, 1980) and hence it is conceivable that the inability of SD15 to transform may be related to any one of several possibilities and not necessarily a consequence of loss of active pp60$^{c\text{-}src}$ association. The mutant 1387T codes for a truncated middle-T which lacks the ability to associate with cellular membrane fractions, a function which is necessary for transformation. Further, there are a number of mutants such as d123, d1015, d12208, RX2 and pTH which retain *in vitro* kinase activity but are nevertheless transformation defective (Courtneidge and Smith, 1984; Templeton *et al.*, 1984; Markland *et al.*, in preparation). An approach to examine whether complexed middle-T:pp60$^{c\text{-}src}$ is relevant to transformation is to attempt specifically to construct mutants of middle-T which affect their ability to associate stably with pp60$^{c\text{-}src}$ and then assay for their ability to transform. This may be accomplished by introducing mutations into the region of middle-T which is involved in pp60$^{c\text{-}src}$ binding. If the pp60$^{c\text{-}src}$ tyrosine kinase is involved in transformation by middle-T, the prediction is that all mutants of middle-T which severely affect pp60$^{c\text{-}src}$ binding would abolish transformation and the phenotype, transformation-competent, kinase-inactive would be impossible to produce. Further, an analysis of the biological significance of pp60$^{c\text{-}src}$ association with middle-T would also aid in confirming and establishing the presence of this complex in lysates of polyoma-transformed cells. Although it is widely accepted that middle-T associates with pp60$^{c\text{-}src}$ *in vitro*, there is no compelling evidence to date to indicate that the complex exists *in vivo*. Hence, although studies of virus mutants demonstrate a clear correlation between middle-T phosphorylation on tyrosines *in vitro* and cell transformation, there is little or no detectable difference in phosphotyrosine con-

tent on middle-T or on cellular proteins in cells transformed by polyoma virus (Sefton *et al.*, 1980; Segawa and Ito, 1982).

There is increasing evidence to suggest that the site on middle-T to which pp60$^{c\text{-}src}$ binds lies within the amino-terminal portion of middle-T. A monoclonal antibody directed against the amino-terminal 20% of middle-T (Dilworth and Griffin, 1982) is unable to recognise the middle-T:pp60$^{c\text{-}src}$ complex suggesting that this determinant is hidden in complexed but not free middle-T. Also a large number of deletion mutants which lack carboxy-terminal portions of middle-T retain associated kinase activity (Smith and Ely, 1983; Courtneidge and Smith, 1984; Templeton *et al.*, 1984; Markland *et al.*, in preparation) whilst mutants which affect the amino half of middle-T cause a defect in this activity (Carmichael and Benjamin, 1980; Templeton and Eckhart, 1984).

Of particular interest is the middle-T encoded by the hr-t mutant, NG59, which is transformation defective, lacks *in vitro* kinase activity and stably associated middle-T:pp60$^{c\text{-}src}$ (Carmichael and Benjamin, 1980; Courtneidge and Smith, 1983, 1984). The NG59 mutant codes for an altered middle-T that differs from the wild-type by a G to A transition at nucleotide 535 resulting in the conversion of aspartic acid 179 to asparagine and an adjacent insertion of the sequence ATA, coding for an in-frame isoleucine between residues 178 and 179. These changes represent the smallest mutation described to date which eliminates both the tyrosine kinase and transforming activity of middle-T. The NG59 lesion therefore defines a possible pp60$^{c\text{-}src}$ binding site. Experiments were hence designed to construct more mutants of middle-T to ascertain the involvement of this region in pp60$^{c\text{-}src}$ binding and ultimately of the contribution of this association to transformation by polyoma virus. Mutagenesis of this region was approached in three ways: (i) linker insertion/deletion mutants were created in the region of DNA coding for amino acids 157 – 185, the area flanking the NG59 mutation, (ii) site-specific mutagenesis using specific synthetic oligonucleotides was used to separate the transition and insertion components of the NG59 mutation, (iii) site-directed mutagenesis using a mixture of oligonucleotides of pre-determined degeneracy was used to generate a family of defined point mutants centred around the NG59 site. The phenotype of the resulting middle-T species was determined.

Results

Construction of mutants

All the polyoma mutations were introduced into a plasmid, pAS101, which contains the 2200-bp *Bam*HI-*Eco*RI early region fragment of wild-type polyoma virus inserted in pAT153 (Oostra *et al.*, 1983). The insert which is capable of directing the synthesis of polyoma middle-T, small-t and a fragment of large-T can transform the growth of Rat-1 cells in culture when introduced by calcium phosphate transfection.

Deletion mutagenesis of the region surrounding the NG59 lesion. Linker insertion/deletion mutants were constructed as described in Materials and methods using two mutant plasmids, XD9 and XD170, which contain at the borders of the region to be mutated [nucleotides (XD170) 657 – (XD9) 789], a synthetic oligonucleotide *Xho*I linker (Figure 1). Two series of exonuclease-digested products, the XA mutants and XB mutants, generated by *Bal*31 treatment of XD170 and XD9 respectively, were combined to create small overlapping deletion mutants spanning the NG59 lesion (Table I). The combination of *Bam*HI-*Xho*I fragments from the XA and XB mutants was chosen such that the reading frame of middle-T was preserved. The precise nature of the deletions

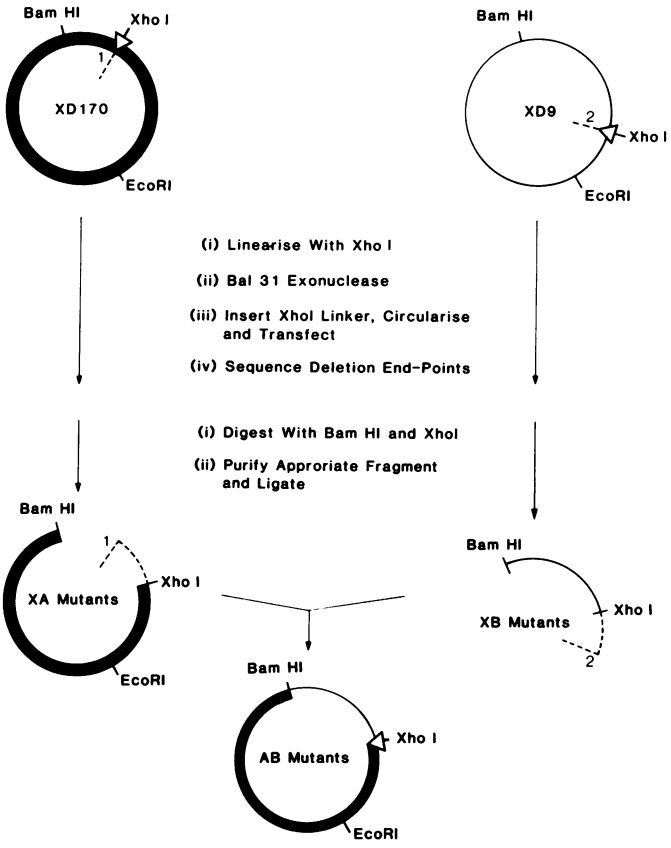

Fig. 1. Construction of linker insertion/deletion mutants. The mutant plasmids AB1 – AB5 were constructed by combining fragments of selected *Bal*31 exonuclease digestion products so as to create small in-frame deletion mutants between the sites of linker insertion in the plasmids XD170 and XD9. In this figure, polyoma virus-derived sequences are bounded between the *Bam*HI and *Eco*RI sites and contain the *Xho*I linker.

Table I. Summary of deletion mutants

(a) Bal31 exonuclease digestion of XD170 and XD9. Last polyoma nucleotide to 3′ side of *Xho*I linker (XA) and 5′ side of *Xho*I linker (XB) (both in the sense of polyoma virus early mRNA) are indicated.

XD170 : 657	XD9 : 789
XA39 : 705	XB50 : 719
XA42 : 708	XB1 : 715
XA8 : 725	XB39 : 686
XA4 : 726	XB38 : 674
XA11 : 739	XB7 : 652
	XB42 : 642

(b) Combination of Bal31 exonuclease digestion products of XD170 and XD9 which preserves the normal middle-T reading frame. Last polyoma nucleotides either side of the 8-bp *Xho*I linker are indicated.

AB1 (XB1-XA4)	: 715 – 726
AB2 (XB39-XA8)	: 686 – 725
AB3 (XB38-XA8)	: 674 – 725
AB4 (XB42-XA42)	: 642 – 708
AB5 (XB42-XA39)	: 642 – 705

was verified by DNA sequencing using the oligonucleotide-primed dideoxy method (Smith, 1980). Such deletion mutants (the AB series of mutants) contained amino acids encoded by the linker sequence at the site of the deletion. Five such linker insertion/deletion mutants (AB1 – AB5) are shown in Table I which collectively delete DNA sequences of the polyoma middle-T antigen encoding for the amino acids 157 – 185. These were tested for their ability to transform cells in culture.

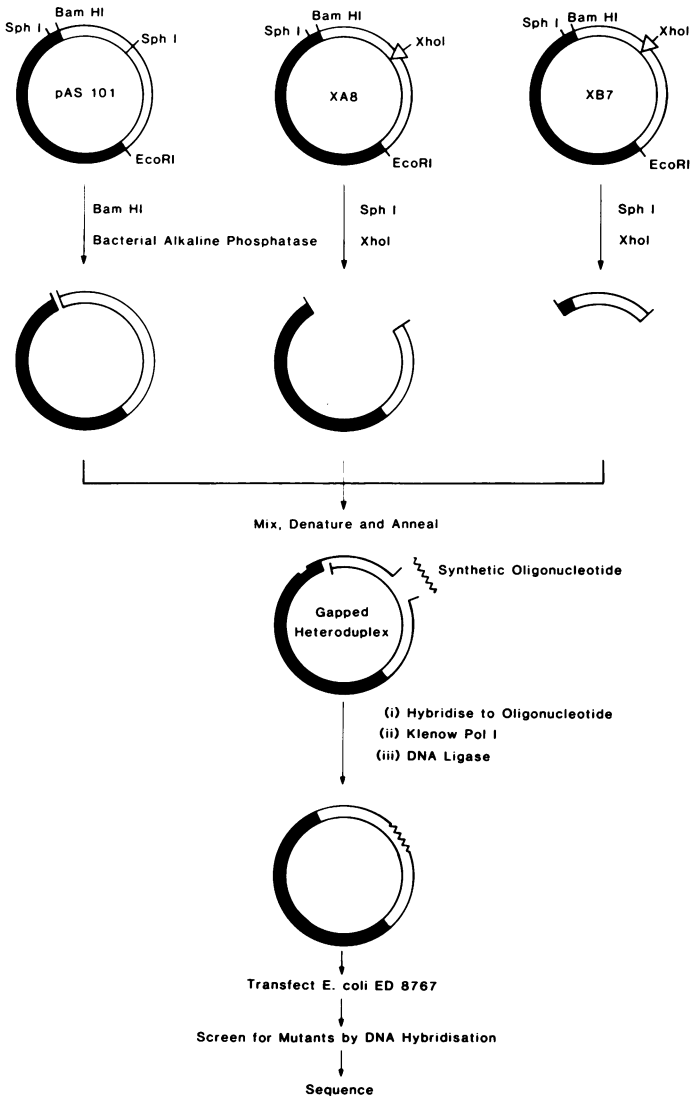

Fig. 2. The procedure for oligonucleotide-directed mutagenesis using a gapped heteroduplex molecule. The gapped heteroduplex was constructed by annealing appropriate restriction fragments from the wild-type plasmid pAS101 and two linker insertion/deletion mutants, XA8 and XB7. The resultant molecule contains a 73-nucleotide gap to which the oligonucleotides or mixture of oligonucleotides were hybridized. Following incubation with Klenow polymerase I and DNA ligase, the material was used directly for transfection. In the figure, plasmid DNA sequences are shaded whereas the polyoma virus-derived sequences are not.

Oligonucleotide-directed mutagenesis to introduce point mutations into the region 701-712. To ascertain the effect of the individual mutations in the NG59 lesion and of the surrounding region on the transforming activity of middle-T and its ability to form a complex with pp60$^{c\text{-}src}$, both oligonucleotide-directed and mixed oligonucleotide mutagenesis were employed. Mutagenesis employing either a single or a mixture of synthetic oligonucleotides was performed using a gapped heteroduplex molecule. As shown in Figure 2, a heteroduplex molecule was formed between wild-type plasmid DNA and two restriction fragments from two linker insertion/deletion mutants. The resultant heteroduplex molecule generates a gap spanning nucleotides 652 – 725 to which the synthetic oligonucleotides could be hybridized.

To generate separately the two lesions of NG59, two 17-residue long oligonucleotides synthesized as described by Edge *et al.*

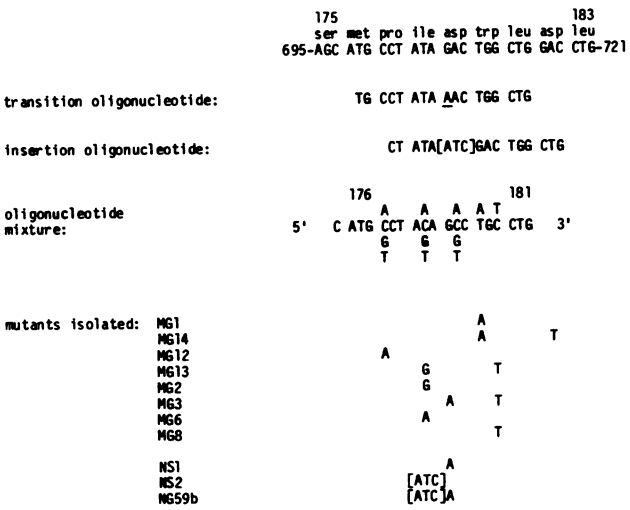

Fig. 3. Mutants obtained using oligonucleotide-directed mutagenesis. The synthetic oligonucleotides used to generate the transition and insertion lesion of NG59 together with the degenerate oligonucleotide mixture are shown above. The nucleotide sequence alterations in the various mutant plasmids are displayed. The corresponding changes in the amino acid sequence of middle-T are presented in Table II.

(1981) were used as mutagens (Figure 3). The sequences of both oligonucleotides were in the same sense as early mRNA. The oligonucleotide TGCCTATAAACTGGCTG corresponding to nucleotides 699 – 715 of polyoma virus DNA and containing a single mismatch at residue 707 (where G is replaced by an A) was used to generate a G to A transition causing amino acid 179, aspartic acid, to be converted to asparagine (NSl). To generate the other component of the NG59 lesion, the oligonucleotide CTATAATCGACTGGCTG corresponding to nucleotides 701 – 715 and containing an insertion of three nucleotides ATC, between residues 706 and 707, was used as mutagen. The insertion introduces an additional isoleucine between amino acids 178 and 179 of polyoma middle-T (NS2).

To generate a large number of point mutants surrounding the NG59 lesion, a mixture of oligonucleotides of pre-determined degeneracy was utilized as mutagens. The mixture of oligonucleotides, each of 19 residues, was synthesized on a single resin. The composition of the mixture of synthetic oligonucleotides was designed such that each of the residues in the sequence 177-Pro-Ile-Asp-Trp-180 could potentially be coverted to any of two or three different amino acids (Figure 3). This was achieved by adding an equimolar amount of all four nucleotides at selected positions along the length of the oligonucleotide during synthesis. At two positions, nucleotide numbers 710 and 712, an equimolar amount of only two nucleotides were used. This was to prevent the possible formation of termination codons.

Mutagenesis was performed by annealing the mixture of oligonucleotides into the gapped heteroduplex molecule constructed as described above. Following transfection, plasmids bearing mutations in this region were distinguished from wild-type by hybridizing with a ^{32}P-labelled 17-residue oligonucleotide of wild-type sequence. Following hybridization at room temperature, filters were washed at increasing temperatures (Wallace *et al.*, 1979). Hybrid plasmids of mutants bearing the insertion of three nucleotides were shown to be stable up to 37°C, whilst those with double point mutants were stable to 40°C and single point mutants, 45°C. By contrast hybrids with wild-type plasmids were stable up to 58°C. Plasmids which formed hybrids with reduced stability were further characterized by DNA sequencing. In the

Table II. Mutations surrounding the NG59 region of polyoma virus middle-T antigen

176	Met	Pro	Ile	Asp	Trp	Leu	Asp	Leu	183		Transformation % (1)	(2)	Kinase	Increased pp60$^{c\text{-}src}$ activity (3)	(4)	Predicted β-turn
		β-turn														
										pAS101	100	100	+	19.5	11.1	+
				Ile[b]	Asn					NG59b	<1	<1	−	0	0	−
					Asn					NS1	160	99	+	16.5	8.7	+
				Ile[b]						NS2	<1	<1	−	0	0	−
					Arg					MG1	<1	<1	−	0	0	−
					Arg		Tyr			MG14	<1	<1	−	0	0	−
		Thr								MG12	24	<1	+	5.6	3.7	−
			Arg		Cys					MG13	22	<1	+	4.9	2.6	+
			Arg							MG2	115	83	+	14.2	8.1	+
				Asn	Cys					MG3	85[a]	79	+	N.D.	N.D.	+
			Lys							MG6	174[a]	107	+	N.D.	N.D.	+
					Cys					MG8	197[a]	159	+	N.D.	N.D.	+

Transformation assay, (1) NIH3T3 cells expressing both neomycin and mutant middle-T antigens were suspended in 0.3% agar. The values represent the number of colonies of transformed cells growing in the soft agar as a percentage of the number obtained using the wild-type plasmid, pAS101.
[a]The assay was performed using transformed cells grown from foci.
(2) Plasmid DNA (1 μg) was transfected into semi-confluent Rat-1 cells by the calcium phosphate method. The numbers represent the mean of duplicate assays expressed as a percentage of the number of foci induced by pAS101 under the same conditions. 1 μg of pAS101 DNA gave 130 foci 14 days post-transfection.
Quantitative kinase assays were performed using either (3) a peptide antibody specific for pp60$^{c\text{-}src}$ or with (4) hamster anti-T serum.
[b]Insertion.
N.D.: not determined.

case of the insertion mutant (NS2), it was possible to screen for the mutation by the generation of a new *Taq*I restriction enzyme site.

With the mixture of oligonucleotides as mutagen, a total of 13 mutants were obtained, 10 of which contained a single point mutation and three contained alterations at two positions. However, five pairs of the single point mutants were duplicates. The mutations occurred at positions which corresponded to the degeneracies in the oligonucleotide mixture except for two mutant plasmids, MG3 and MG14. These both contain mutations at positions which should not theoretically have been introduced by the oligonucleotide mixture used. All the mutants isolated were further characterised by sequencing across the whole of the gap (nucleotides 652 – 725) used to generate this series of mutants and also by restriction enzyme digestion with several multi-cutting enzymes to ensure the absence of other mutations elsewhere in the sequence.

Deletion-loop mutagenesis to generate the NG59 lesion. Since the viral form of the hr-t mutant, NG59, has only been sequenced in part, and to ensure that the reported lesion represents the only mutation, we did not clone out middle-T from NG59 mutant polyoma virus but, instead, this lesion was engineered into the plasmid, pAS101. This was achieved by deletion-loop mutagenesis performed on a heteroduplex formed between the insertion mutant, NS2 and the linker insertion/deletion mutant, AB2. The mutant AB2 was chosen because it affords the smallest deletion spanning the target region (nucleotides 686 – 725). The heteroduplex formed contains a single-stranded region of DNA corresponding to the sequence missing in the deletion mutant, in this case harboring the insertion lesion of NS2. To introduce the transition of NG59, the sequence within the deletion loop was subjected to mild chemical mutagenesis using sodium bisulphite with the aim of mutagenising only the nucleotide 707.

Following transfection of the bisulphite-treated heteroduplexes, colony hybridization was performed using a radiolabelled synthetic oligonucleotide, CTATAATCGACTGGCTG, which corresponds to the insertion lesion in NS2. Plasmids for which hybrids were stable to between 40 and 45°C (representing single or double point mutants) were further characterised for the loss of a *Taq*I site and also by DNA sequencing. One such plasmid harboured a single G to A transition at nucleotide 707. The resultant mutant which contains both the insertion and transition mutations was designated NG59b (Figure 3). It should be noted that whilst in the original NG59 mutant, the insertion consists of the sequence ATA, in NG59b, the sequence is ATC. However, both codons translate to isoleucine.

Biological properties of mutant polyoma virus middle-T antigens

The amino acid sequence in the region of the NG59 mutation of wild-type and the variant middle-T species is shown in Table II. The middle-T mutants were assayed for their ability (i) to transform Rat-1 cells in culture, (ii) to associate with pp60$^{c\text{-}src}$ and (iii) to stimulate the pp60$^{c\text{-}src}$ tyrosine kinase activity *in vitro*.

Transforming activity. The transforming activity of the mutant plasmids was assayed by the formation of dense foci on a monolayer of Rat-1 cells following transfection by the calcium phosphate method (Graham and van der Eb, 1973). The formation of dense foci under these conditions is middle-T dependent (Triesman *et al.*, 1981). Wild-type plasmid DNA (pAS101) gave up to 130 foci after 14 days following transfection of 1 μg plasmid DNA onto ~ 10^6 cells. Parallel experiments using salmon sperm DNA, pAT153 or a phase-altering mutant (XB50-XA11) encoding a truncated middle-T product (185 amino acids) failed to produce any foci even after a prolonged incubation (5 weeks).

When the in-phase linker insertion/deletion mutants, AB1 – AB5, were tested in the same manner, all failed to produce foci. This indicated that the region surrounding the NG59 lesion was sensitive to mutation and was required for transforming activity by the middle-T antigen. However, when the point mutants created by site-directed mutagenesis were assessed for transforming activity using the same assay, some of the mutants possessed the ability to induce foci while others did not (Table II).

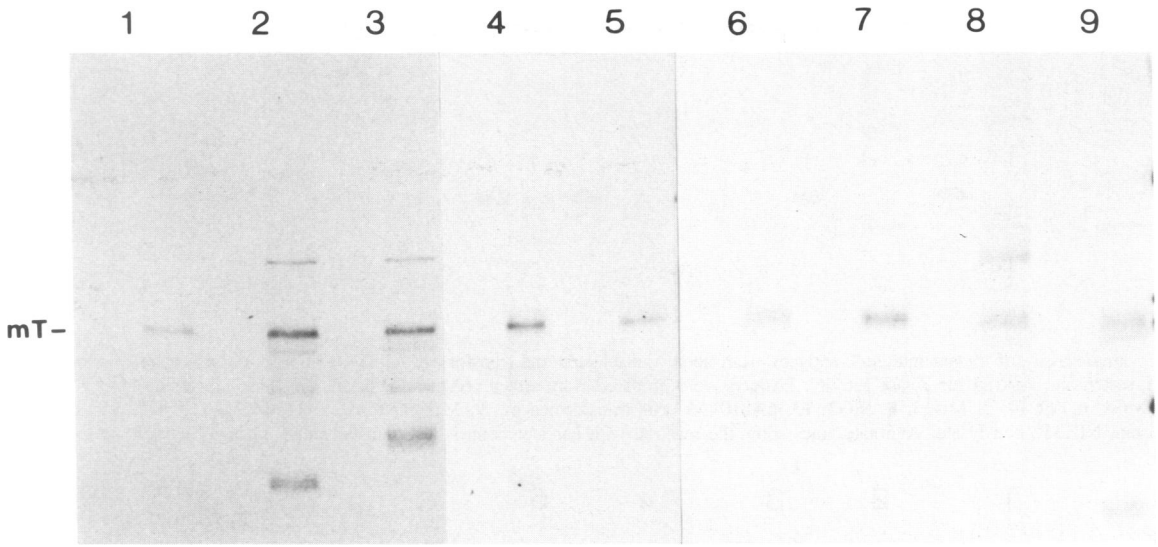

Fig. 4. Immunoprecipitation from neomycin-resistant NIH3T3 cells containing mutant middle-T antigens. The cells were radioactively labelled with [^{35}S]methionine for 2 h and the extracts immunoprecipitated using rat control and rat anti-T serum. The labelled products were separated on a 10% polyacrylamide gel which was dried and then fluorographed. Extracts used were from neomycin-resistant cells expressing the following middle-T variants, **1**, NG59b; **2**, NS1, **3**, NS2; **4**, MG1; **5**, MG12; **6**, MG13; **7**, MG14; **8**, MG2; **9**, pAS101. Alternate lanes show the reaction with normal rat and rat anti-T serum. The position of middle-T antigen is indicated on the left margin. Other bands present on the fluorograph probably represent the small-t and truncated large-T antigens and possibly proteolytic degradation products of the middle-T antigen.

The mutant plasmid NG59b was transformation-defective confirming the previous observation using mutant virus (Carmichael and Benjamin, 1980). Using plasmids with the two separate lesions of NG59, it was found that the insertion was responsible for the loss of transforming activity since the transition mutant, NS1, was able to induce foci at wild-type frequency whilst NS2 (which contains the insertion component) was totally defective. Four mutants, MG2, MG3, MG6 and MG8 all exhibited transforming activity similar to wild-type. No differences in the time of appearance of foci or morphology of the transformed cells were detected. The mutants MG1, MG12, MG13 and MG14, however, consistently failed to induce foci even when larger amounts of DNA (10 μg) were used in the transfections. To ensure that this phenotype was a direct consequence of the mutations determined, two of the non-transforming mutants MG1 and NS2 were reverted to wild-type. This was achieved using the same protocol for a gapped heteroduplex (Figure 2) except pAS101 was replaced by either MG1 or NS2 and the mutagenising oligonucleotide was of wild-type sequence. Revertants of MG1 and NS2 isolated in this manner exhibited transforming activities similar to that of wild-type, indicating the mutations in MG1 (Trp180 to Arg) and NS2 (insertion of isoleucine between Ile178 and Asp179) were indeed responsible for the observed phenotype.

To confirm that the loss of transforming activity was a direct consequence of the point mutations and not of loss of expression of a middle-T antigen, cell lines expressing the non-transforming mutant proteins were isolated. These were obtained by co-transfecting into NIH3T3 cells, mutant plasmid DNAs, and another plasmid pSV2neo which confers resistance to the antibiotic neomycin on eukaryotic cells. Neomycin-resistant clones were selected and analysed for the presence of the mutant middle-T antigen by isotopic labelling followed by immunoprecipitation using a rat anti-T serum.

Cells expressing middle-T antigen encoded by the mutants NG59b, NS1, NS2, MG1, MG12, MG13, MG14 and MG2 obtained using neomycin selection (Figure 4) were tested for transforming activity by their ability to grow as anchorage-independent colonies in soft agar (MacPherson and Montagnier, 1964). Different numbers of cells were suspended in semi-solid medium (0.3% agar) and the appearance of colonies of transformed cells was noted. Under these conditions, neomycin-resistant NIH3T3 cells did not proliferate, whereas >80% of the resuspended cells gave rise to colonies when pAS101-3T3 cells were used. In agreement with the transformation data obtained using calcium phosphate, no significant difference from wild-type behaviour was detected with the mutant cells, NS1-3T3 and MG2-3T3, either in the number or size of colonies detected or their time of appearance. However, in contrast, MG12-3T3 and MG13-3T3 gave rise to a small number of colonies of similar size to wild-type (Table II). The time of appearance of colonies was also delayed by 3–5 days. This indicates the middle-T antigen encoded by both the mutants MG12 and MG13 had weak transforming activity. Possible reasons for the ability of these two mutants to transform weakly using the soft agar assay but not in the calcium phosphate method could be the difference in the cell lines used, NIH3T3 being less stringent than Rat-1 for transformation, or the higher expression of the middle-T protein in the neomycin-selected cell lines. Cell lines transformed by MG3, MG6 and MG8 and grown up from a number of randomly selected foci gave rise to wild-type-like colonies when tested for growth in agar (Table II). The neomycin-selected cells, NG59b-3T3, NS2-3T3, MG1-3T3 and MG14-3T3 failed to proliferate under these conditions.

Association of mutant middle-T antigens with pp60$^{c\text{-}src}$. Immunoprecipitates containing transformation-competent middle-T species have an associated tyrosine kinase activity. When extracts from polyoma virus-transformed cells are assayed for this activity using hamster anti-T serum, middle-T becomes phosphorylated (Smith *et al.*, 1979) whereas, using rat anti-T serum, either middle-T or immunoglobulin molecules or both become phosphorylated (Smith *et al.*, 1979, 1980). The kinase activity is believed to be a consequence of the association of middle-T with pp60$^{c\text{-}src}$. This association is monitored by the

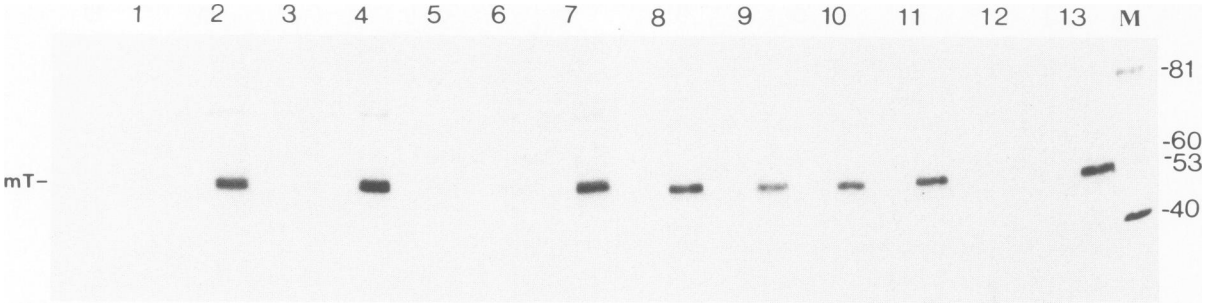

Fig. 5. *In vitro* kinase assay of mutant middle-T antigens from neomycin-selected and transformed cells. Unlabelled extracts were immunoprecipitated with hamster anti-T serum and assayed for kinase activity. Extracts were prepared from either neomycin-resistant cell lines expressing, **1**, NG59b; **2**, NS1; **3**, NS2; **4**, MG12; **5**, MG1; **6**, MG14; **7**, MG13; **8**, MG2; **13**, pAS101 or cells transformed by, **9**, MG3; **10**, MG6; **11**, MG8. **Track 12** was a control assay of neomycin-resistant NIH3T3 cell lysate. Alternate lanes show the reaction with hamster control and hamster anti-T serum. M, mol. wt ($\times 10^{-3}$).

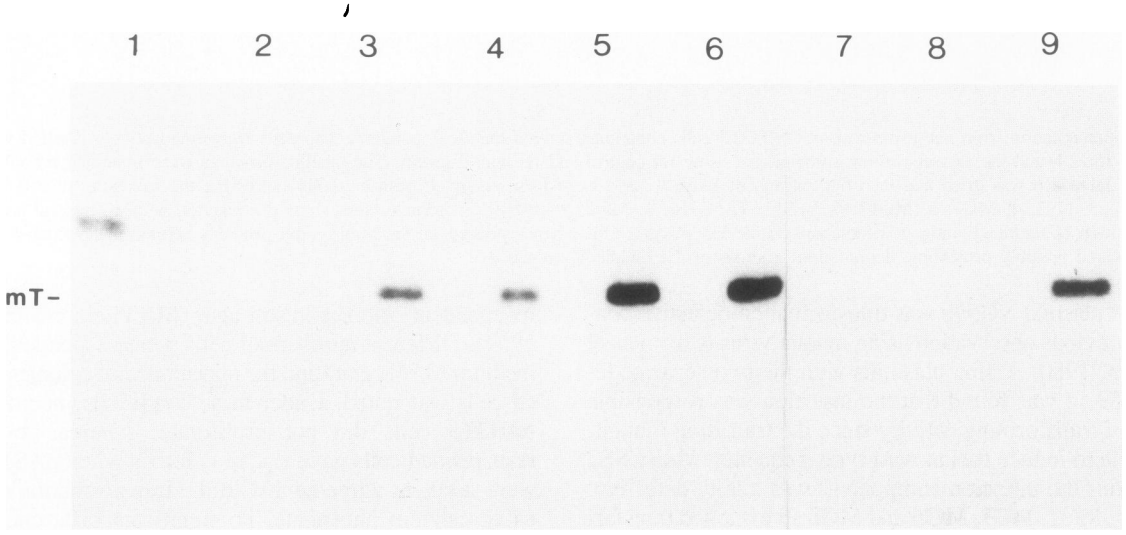

Fig. 6. *In vitro* kinase assay of immunoprecipitates from neomycin-resistant cell lines expressing mutant middle-T antigens using a peptide antibody specific for the carboxy terminus of pp60$^{c\text{-}src}$. Cell lysates were adjusted to the same protein concentration and the phosphorylated reaction products of the immune complex kinase assays were analysed on a 10% polyacrylamide gel. **Lane 1,** MG1; **2**, MG14; **3**, MG12; **4**, MG13; **5**, pAS101; **6**, NS1; **7**, NS2; **8**, NG59b; **9**, MG2. The position of middle-T antigen is indicated on the margin.

ability of immunocomplexes formed using antibodies raised specifically towards pp60$^{c\text{-}src}$ to phosphorylate middle-T in the *in vitro* kinase assay (Courtneidge and Smith, 1984). As previously noted, there are no known transformation-competent mutants of polyoma which lack a middle-T associated tyrosine kinase activity. As the series of mutants isolated above displayed phenotypes ranging from transformation-competent to transformation-defective, the kinase activity of the mutants and their ability to associate with pp60$^{c\text{-}src}$ were investigated.

Figure 5 shows the result of a kinase assay on immunoprecipitates formed with hamster anti-T sera using lysates of NIH3T3 and Rat-1 cells expressing transformation-competent or defective middle-T species. All cells which expressed mutant middle-T species with transforming activity displayed kinase activity in this assay. The middle-T antigens encoded by MG12 and MG13 which were shown to possess only weak transforming activity also exhibited associated kinase activity. These data support the notion that all transformation-competent mutants of polyoma virus, irrespective of the degree of transformation, possess kinase activity *in vitro*. Non-transforming middle-T mutants consistently failed to display phosphate acceptor activity when hamster anti-T sera was used or to phosphorylate immunoglobulin molecules using rat anti-T sera (data not shown).

To determine whether the mutants associated with pp60$^{c\text{-}src}$, a similar kinase assay was performed but using a peptide antibody specific for the carboxy terminus of pp60$^{c\text{-}src}$. All but the four non-transforming, kinase-deficient mutants of middle-T (NG59b, NS2, MG1 and MG14) were co-immunoprecipitated with anti-c-*src* sera and were phosphorylated *in vitro*. This again demonstrates the strong correlation between the ability of middle-T to transform, to possess kinase activity and to associate stably and actively with pp60$^{c\text{-}src}$. Figure 6 shows a selection of mutants assayed using anti-c-*src* sera. For this assay, the amount of lysates have been normalized with respect to protein concentration. As the cells contain approximately equivalent amounts of middle-T (Figure 4) and as pp60$^{c\text{-}src}$ levels are unaffected by transformation by polyoma virus (Bolen *et al.*, 1984), the immunoprecipitates can be assumed to contain approximately equal amounts of middle-T antigen and pp60$^{c\text{-}src}$. Under these conditions, it can be seen that the mutants encoded by MG12 and MG13 which have weak transforming activity display a reduction in kinase activity when compared with wild-type middle-T (pAS101) or transformation-competent mutants (NS1, MG2).

In summary, the results using both hamster anti-T serum and anti-c-*src* serum demonstrate (i) that all transformation-positive middle-T species have associated kinase activity and (ii) that this

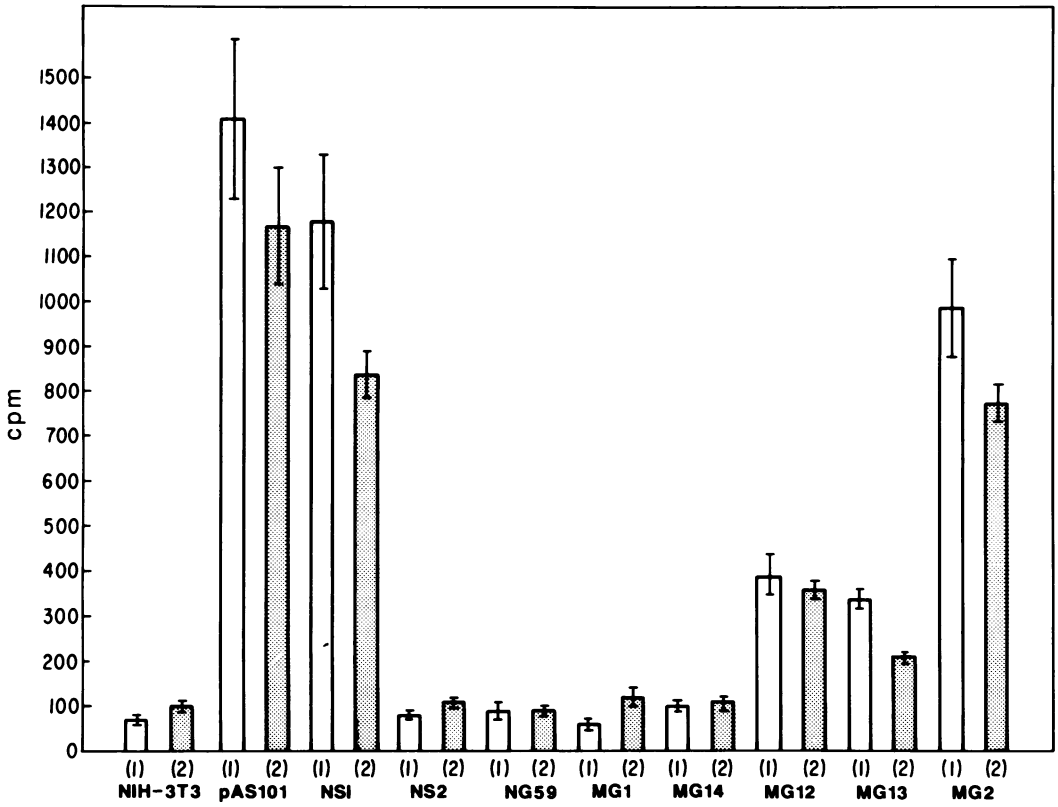

Fig. 7. Quantitation of the kinase activity of pp60$^{c\text{-}src}$. Lysates of neomycin-resistant NIH3T3 cells containing mutant middle-T antigens were immunoprecipitated with **(1)** anti-c-*src* sera and **(2)** hamster anti-T sera and the kinase assay performed in the presence of enolase. The region of the gel containing enolase was excised and counted in a scintillation counter to determine the extent of the reaction. As negative controls, immunoprecipitates made either with anti-peptide antibody pre-blocked with specific peptide or with hamster control serum were also analysed and these values subtracted from the experimental values. Depicted are the number of counts in enolase after a 4 min reaction at 30°C. Error bars show the mean of values obtained in triplicates.

activity parallels the ability of the middle-T species to form a stable and active complex with pp60$^{c\text{-}src}$. The middle-T antigen encoded by the mutant MG1 reduces the smallest mutation which renders middle-T defective in transformation and associated kinase activity to a single amino acid change.

Activation of the pp60$^{c\text{-}src}$ kinase activity by mutant middle-T antigens. The interaction between middle-T encoded by transformation-competent strains of polyoma and pp60$^{c\text{-}src}$ results in a stimulation of the pp60$^{c\text{-}src}$ kinase specific activity in both polyoma-infected and polyoma-transformed rodent cells (Bolen *et al.*, 1984; Courtneidge, 1985). This increase in kinase activity can be monitored quantitatively by the increased phosphorylation of exogenous substrates such as casein (Bolen *et al.*, 1984) or enolase (Courtneidge, 1985). As the ability of MG12 and MG13 to phosphorylate middle-T in immunoprecipitates and to transform was intermediate between wild-type and defective, the ability of all the mutants isolated to stimulate the activity of pp60$^{c\text{-}src}$ was further investigated.

Using samples of lysates from the neomycin-selected mutant middle-T cell lines normalised with respect to protein concentration, pp60$^{c\text{-}src}$ was immunoprecipitated using anti-c-*src* or hamster anti-T sera and kinase assays performed. The quantitative results of these assays using enolase as the substrate are depicted graphically in Figure 7 and summarised in Table II. The major phosphorylated band observed in these assays using either hamster anti-T serum or anti-c-*src* serum co-migrated with Coomassie Blue-stained enolase. Using anti-c-*src* sera, the level of phosphorylation in immunoprecipitates prepared from lysates of NIH3T3 cells containing wild-type middle-T antigen was found to be increased 20-fold over that observed in control NIH3T3 cells. Increased levels of kinase activity (14- to 17-fold) were also detected for two transforming mutants (NS1-3T3; MG2-3T3). However, lysates containing middle-T antigen encoded by the transformation-defective mutants (NG59-3T3; NS2-3T3; MG1-3T3; MG14-3T3) were found to have levels of kinase activity indistinguishable from that in normal NIH3T3 cell lysates. In addition, a similar analysis of the immunoprecipitates prepared from MG12-3T3 and MG13-3T3 lysates, which contain middle-T antigens with weak transforming activity, showed only a 4- to 5-fold increase in enolase phosphorylating activity over normal NIH3T3 cell lysates. To check that this activation was a direct consequence of pp60$^{c\text{-}src}$ complexed with middle-T, the assays were performed using hamster anti-T serum. Analyses of these immunoprecipitates revealed a pattern of kinase activation identical to that observed with anti-c-*src* serum except the fold increase in kinase activity was lower in all cases (Table II).

Taken together, the results with the present mutants confirm that binding of middle-T to pp60$^{c\text{-}src}$ results in an increase in kinase activity only in the case of transformation-competent mutants. Furthermore, using enolase as an exogenous substrate, the data obtained with MG12 and MG13 confirm that with these mutants the reduced ability to phosphorylate middle-T in the initial kinase assay was a consequence of a reduction in the kinase activity rather than a decrease in phosphate acceptor activity. The

strong correlation between transforming activity and kinase specfic activity further supports a role for the pp60$^{c\text{-}src}$ complex in transformation by polyoma virus.

Prediction of secondary structure of the middle-T mutants. When the amino acid sequence of the mutant middle-T species was subjected to computer-assisted secondary structure analysis as described by Chou and Fasman (1978), it was found that residues 177 – 180 are predicted to form a β-turn in the wild-type middle-T protein. Further analysis revealed a surprising correlation between the predicted presence of the β-turn, the presence of associated activated pp60$^{c\text{-}src}$ and the transforming activity of the mutant middle-T species (Table II). Mutants coding for a middle-T antigen which disrupted the predicted β-turn in this region lose the ability to associate stably with pp60$^{c\text{-}src}$ and to transform. Of the mutants isolated above, the only exception to this rule was the mutant encoded by MG12 which was predicted to lose the β-turn conformation but still retained weak transforming activity.

Discussion

A generally accepted hypothesis for transformation by polyoma virus is that a complex between the middle-T antigen and pp60$^{c\text{-}src}$ plays an essential role. The binding of middle-T to pp60$^{c\text{-}src}$ results in an altered pp60$^{c\text{-}src}$ with enhanced tyrosine kinase activty and, this in turn, it is supposed, leads by an unknown mechanism to an increase in cell proliferation (Courtneidge and Smith, 1984; Bolen *et al.*, 1984; Courtneidge, 1985; Yonemoto *et al.*, 1985). In an attempt to investigate further the significance of the complex in the transformation process and to probe for the pp60$^{c\text{-}src}$ binding site on middle-T, mutations were made in the region surrounding the NG59 lesion since previous studies had shown this mutant lacked transforming and kinase activities.

A series of overlapping deletions adjacent to the NG59 site were transformation defective indicating the region is required by middle-T in this process. The series of point mutants introduced between amino acids 177 – 180 can be categorised into three different groups based on their respective biological and biochemical properties. Of the 11 different point mutants isolated, a group of five mutants displayed essentially a wild-type phenotype with regard to transforming activity, associated pp60$^{c\text{-}src}$ kinase activity and their ability to phosphorylate an exogenous substrate. A second group comprising four mutants were totally defective in all the above activities and, finally, two mutants were characterised which possessed activities at intermediate levels. The finding here that mutants unable to associate stably with pp60$^{c\text{-}src}$ lack kinase activity is consistent with the suggestion that the middle-T antigen does not possess intrinsic kinase activity (Courtneidge and Smith, 1984; Schaffhausen *et al.*, 1985). The perfect correlation between the ability of middle-T to transform and possess associated pp60$^{c\text{-}src}$ kinase activity supports the hypothesis that the formation of an active complex between the two proteins is an essential component of middle-T transformation. The mutants described here are the most extensive to date which were specifically constructed to test this hypothesis. Only those mutants mapping in this region which displayed pp60$^{c\text{-}src}$ kinase activity were transformation competent whilst all the transformation-defective mutants were totally devoid of this activity. The inability to generate a mutant of middle-T which lacked kinase activity but retains transforming activity confirms the suggestion that pp60$^{c\text{-}src}$ association is central to polyoma virus middle-T-mediated transformation. Recently, Raptis *et al.* (1985) showed, using the dexamethasone-inducible promoter from the mouse mammary tumor virus, that the expression of transformation parameters does not correlate with the total amount of middle-T expressed but with the level of middle-T phosphorylated by pp60$^{c\text{-}src}$ in the immunocomplex. Taken together with the earlier observations using monoclonal and peptide antibodies (Bolen *et al.*, 1984; Courtneidge and Smith, 1984), the data presented here thus appear to establish beyond any reasonable doubt that a complex between middle-T and pp60$^{c\text{-}src}$ exists in lysates of polyoma virus-transformed cells.

The results also show that there is a close correlation between the transforming activity of middle-T, as judged by the number of foci induced and the pp60$^{c\text{-}src}$ tyrosine kinase specific activity. Mutants of middle-T which have wild-type transforming activity possess high pp60$^{c\text{-}src}$ kinase activity whilst non-transforming mutants only have basal levels. This correlation is best exemplified by the mutant middle-Ts encoded by MG12 and MG13, which have corresponding intermediate levels of both activities. The latter mutants provide, perhaps, the best evidence to date for the correlation between pp60$^{c\text{-}src}$ activation and transforming activity. Thus the ability of middle-T to associate with pp60$^{c\text{-}src}$ and to activate its tyrosyl kinase activity remains the best correlation of polyoma virus-induced transformation. It may be suggested from the observations that the transforming activities of the mutants encoded by MG12 and MG13 reflect a reduced ability to activate the kinase specific activity. It is possible these two mutants encode variant middle-T species which have a limited ability to associate actively with pp60$^{c\text{-}src}$. Mutations mapping to the same amino acid, but which encode for a different amino acid and which are nevertheless transformation defective (i.e. MG1), represent middle-T antigens totally defective in this capability. The results also show that of the two mutations present in the NG59 lesion, the insertion of isoleucine is responsible for abolishing transforming and *in vitro* kinase activities whereas the transition event has no effect.

The complete correlation between the ability of the point mutants around the NG59 lesion to associate actively with pp60$^{c\text{-}src}$ and to transform supports the suggestion that the region is involved in the binding and activation of pp60$^{c\text{-}src}$. The importance of this region is demonstrated by the mutant middle-T encoded by MG1 which represents only a single amino acid change (Trp180 to Arg) but which results in both a transformation- and kinase-inactive molecule.

When examined for predicted conformation changes using the Chou and Fasman (1978) analysis, it transpired that the region 177 – 180 was predicted to comprise a β-turn in middle-T. β-Turns are structural features of peptides and proteins that involve four consecutive residues where the polypeptide chain folds back on itself (Crawford *et al.*, 1973). An analysis of all mutants revealed a strong correlation between the presence of a β-turn and the ability of the middle-T mutants to retain transforming activity. It is not clear, however, whether this correlation might result because loss of the β-turn results in a gross conformational change which alters a distant pp60$^{c\text{-}src}$ binding site in middle-T or whether the β-turn itself is a surface feature that is directly involved in the binding to the enzyme. The finding that different point mutations which alter the same amino acid residue into alternative amino acids can give rise to either transformation and kinase active or defective phenotypes is easier to reconcile with the suggestion that this region performs some distant structural role in pp60$^{c\text{-}src}$ binding mediated by the β-turn rather than acts as a direct binding site.

Recently, Bolen and Israel (1985) using monoclonal antibodies

presented evidence that the NG59-encoded middle-T antigen does associate, albeit weakly and unstably, with pp60$^{c\text{-}src}$. In sharp contrast to wild-type middle-T, the NG59 middle-T:pp60$^{c\text{-}src}$ complex was shown to be extremely sensitive to ionic detergents and high concentrations of salt. This suggests that the mutation had severely affected the stable association of pp60$^{c\text{-}src}$ with middle-T hence lending further credence to the suggestion that this region is involved in binding to pp60$^{c\text{-}src}$. Under the conditions used in this study, the weak binding reported by Bolen and Israel (1985) was not detected.

The suggestion that the region around the NG59 mutation contributes to the ability of middle-T to interact successfully with pp60$^{c\text{-}src}$ is further supported by the observation that the sequence in this region (amino acids 167 – 190) is highly conserved between the murine and a recently described hamster polyoma virus middle-T (Delmas *et al.*, 1985). Further, the equivalent position for the predicted β-turn in the murine middle-T is present in the hamster protein as Pro-Val-Asp-Trp. This sequence is also predicted to be a β-turn. The conservation between the two polyoma virus middle-Ts strongly suggests the β-turn and the immediate surrounding region play a significant role in the function of middle-T.

Materials and methods

Mutagenic procedures

Deletion mutagenesis. A modification of the procedure by Kalderon and Smith (1984) was used to introduce deletions into a defined segment of pAS101. Linker insertion/deletion mutants were constructed from two mutant plasmids, XD9 and XD170. These plasmids which have *Xho*I linkers (CCTCGAGG) inserted at the extreme borders of the region to be mutated (nucleotides 657 – 789; numbering system of Tooze, 1981) were further manipulated to generate a series of deletion mutants as outlined in Figure 1. The plasmids XD9 and XD170 were each linearised at their *Xho*I sites and then subjected to *Bal*31 nuclease digestion for increasing periods of time. Prior to recircularisation, *Xho*I linkers were attached to these truncated molecules and their deletion end-points determined by sequencing. In-phase deletion mutants were generated by ligation of appropriate *Bam*HI-*Xho*I fragments of the XD170 (XA mutants) and XD9 (XB mutants) *Bal*31-digested products so as to create chimaeric plasmids analogous to the wild-type plasmid but which contain an *Xho*I linker at the site of the deletion. The particular combinations of *Bam*HI-*Xho*I fragments were chosen such that appropriate deletions were introduced into the whole target region.

Oligonucleotide-directed mutagenesis. Oligonucleotide-directed mutagenesis was performed using the gapped heteroduplex method essentially as described by Oostra *et al.* (1983) and Kalderon *et al.* (1984). A gapped heteroduplex molecule containing a 73-nucleotide gap (nucleotides 652 – 725) was constructed using a wild-type plasmid DNA (pAS101) and two restriction fragments from two linker insertion/deletion mutants as outlined in Figure 2. The linker insertion/deletion mutants XA8 and XB7 are derivatives of pAS101 that contain an 8-bp *Xho*I linker spanning deletions between nucleotides 419 – 725 and 652 – 892, respectively. Appropriate *Sph*I-*Xho*I restriction fragments from XA8 and XB7 were purified by agarose gel electrophoresis and were mixed in equimolar proportions with pAS101 DNA linearised at its unique *Bam*HI site. Following denaturation with alkali and subsequent renaturation (Oostra *et al.*, 1983), the hybridization products were dissolved in 10 μl 1 mM Tris-HCl, pH 8.0, 1 mM EDTA and combined with the appropriate 5′-phosphorylated oligonucleotide or oligonucleotide mixture in 10 μl 50 mM NaCl, 6.6 mM Tris-HCl, pH 7.6, 6.6 mM MgCl, 1 mM dithiothreitol (DTT). To this was added 5 μl of a solution containing the four deoxyribonucleotides at 2.5 mM, plus 2.5 mM ATP, 50 mM NaCl, 5.5 mM Tris-HCl, pH 7.6, 6.6 mM $MgCl_2$ and 1 mM DTT. Following the addition of 1 U large fragment (Klenow) DNA polymerase I and 1000 U T4 DNA ligase, the mixture was incubated for 1 h at 0°C and then continued at 15°C for 17 h. Portions of this reaction mixture were used to transfect *Escherichia coli* ED8767.

Following transfection, plasmids bearing mutations in this region were detected by colony hybridization (Grunstein and Hogness, 1975) using a 17-nucleotide long probe of sequence corresponding to the wild-type gene in this region. Mutants were distinguished from wild-type plasmid by virtue of the thermostabilities of the hybrids formed (Wallace *et al.*, 1979). Plasmids which formed hybrids with reduced thermostability were further characterized by DNA sequencing using the oligonucleotide-primed dideoxy method (Smith, 1980).

Deletion-loop mutagenesis. The NG59 lesion was introduced into pAS101 by deletion-loop mutagenesis as described by Kalderon *et al.* (1982). A heteroduplex molecule was formed between the insertion mutant, NS2 (containing an insertion of nucleotides ATC between residues 706 and 707) and the linker insertion/deletion mutant, AB2 which is missing a 39-bp sequence between nucleotides 686 and 725. Following a mild treatment with sodium bisulphite and transfection into *E. coli* strain K58, mutant plasmids were detected by colony hybridization using a 17-residue long synthetic oligonucleotide (CTATAATCGACTGGCTG) which corresponds to the insertion mutation in the plasmid, NS2. Plasmids harboring the NG59 lesion were confirmed for the presence of the mutations both by the loss of a *Taq*I restriction enzyme site and also by DNA sequencing.

Analysis of polyoma virus middle-T antigen

Transformation assay. Calcium phosphate-DNA precipitates were introduced into Rat-1 cells essentially as described by Graham and van der Eb (1973). Middle-T transformed cells selected as dense foci overgrowing a monolayer of cells were generally visible 14 days following transfection. The same calcium phosphate transfection procedure was used to co-transfect DNAs encoding mutant variants of middle-T and the plasmid, pSV2neo (Southern and Berg, 1982) which confers resistance to neomycin, onto NIH3T3 cells. Transfections were performed using middle-T DNA and pSV2neo DNA in the ratio of 10:1. Neomycin-resistant clones were selected and analysed for the presence of the mutant middle-T antigen by isotopic labelling followed by immunoprecipitation using a rat anti-T serum. Cells which expressed middle-T antigen were assayed for their ability to promote anchorage-independence growth in agar as described by MacPherson and Montagnier (1964).

In vitro kinase assay. Immune-complex kinase assays and quantitative kinase assays using enolase were conducted as previously described (Courtneidge and Smith, 1983, 1984; Courtneidge, 1985).

Radiolabelling of cells and immunoprecipitation. Isotopic labelling of cells with [^{35}S]methionine, preparation of cell lysates, immunoprecipitation of proteins and SDS-polyacrylamide gel electrophoresis were performed as described in Courtneidge and Smith (1983, 1984).

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