THE SEQUENCE TNNCT MODULATES TRANSCRIPTION OF A *DROSOPHILA MELANOGASTER* $tRNA^{Val}_4$ GENE

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ABSTRACT

The transcription efficiency of transfer RNA genes is modulated by sequences contained in their 5'-flanking region. For a $tRNA^{Val}_4$ gene a pentanucleotide with the sequence TCGCT was identified between positions -33 and -38. I have previously proposed that this sequence may be involved in specifically determining the rate of transcription of this gene. A general form of this sequence, TNNCT was found associated with other Drosophila tRNA genes which showed high in vitro transcription efficiency.

To further elucidate the role of TCGCT in tRNA transcription, single and double base-pair changes were created in the sequence TCGCT using site-specific mutagenesis. Mutations in the nucleotides -38T, -35C and -34T showed decreased levels of transcription whereas nucleotide changes at the nucleotides -37C and -36G did not reduce template activity. Therefore the sequence which modulates transcription of the $tRNA^{Val}_4$ gene does have the general form TNNCT. Competition experiments between the Val_4 mutant -38G,-35A and a $tRNA^{Ser}_7$ gene showed the TNNCT mutant to be a better competitor for transcription than the wild type template. Experiments analyzing the time-course of transcription, the effects of temperature and the effects of ionic strength indicated that TNNCT was not involved in determining the efficiency of stable complex formation. It is proposed that the pentanucleotide is probably responsible

for influencing the rate of initiation of transcription. A sequence TGCCT contained in the anticodon stem/loop region of the Val_4 gene was also mutagenized and shown to be involved in complex stability or the elongation of Val_4 tRNAs.

Using deletion analysis of the 5'-flanking sequences of a $tRNA^{Ser}_7$ gene, a second positive transcription regulatory element was delimited. This sequence was also found in the 5'-flanks of the $tRNA^{Val}_4$ and a $tRNA^{Arg}$ gene.

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Abbreviations

BME	2-mercaptoethanol
bp	base pair(s)
BSA	bovine serum albumin
CsCl	cesium chloride
dH_2O	distilled water
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
hr	hour
IPTG	isopropyl-B-D-thiogalactopyranoside
Kb	kilobases
Kd	kilodaltons
L	liter
min	minute
ml	milliliter
Mr	molecular weight
NaCl	sodium chloride
NaOAc	sodium acetate
NH_4OAc	ammonium acetate
PEG	polyethylene glycol
pmol	picomole
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris-Cl	[Tris(hydroxymethyl)aminomethane hydrochloride

tRNA	transfer ribonucleic acid
ul	microliter
Val_4	$tRNA^{Val}_4$
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

INTRODUCTION

I. Class III genes

RNA polymerase III (or polymerase C, M_r ~700,000) is an enzyme composed of two large subunits and a collection of 10-12 smaller components (Huet et al., 1985) responsible for the transcription of a class of RNAs which have become defined as class III genes. The structure and characteristics of RNA polymerase III have been extensively reviewed (Roeder, 1976; Spindler, 1978; Sentenac, 1985).

Genes transcribed by RNA polymerase III include the Alu family of repetitive sequences which are believed to function in regions of DNA replication (Duncan et al., 1979; Jelinek et al., 1980; Fuhrman et al., 1981; Haynes et al., 1981; Hess et al., 1985), the B2 repeated family, whose transcription is enhanced by SV 40 transformation (Singh et al., 1985; Carey et al., 1986a) and human 7SL RNAs which are related to the Alu sequences (Ullu and Weiner, 1985). In addition, a 270 nucleotide RNA from Tetrahymena has been shown to be transcribed by RNA polymerase III in response to heat shock (Kraus et al., 1987). Viral class III genes include Epstein-Barr virus DNA (Jat and Arrand, 1982) and Adenovirus-associated (VA) RNAI and VA RNAII (Thimmappaya et al., 1979; Weil et al., 1979; Wu, 1980; Guilfoyle and Weinmann, 1981). Other genes include the identifier (I.D.) sequences which encode repeated "brain-specific" small RNAs of 82 nucleotides in length. Data suggests that I.D.

sequences present in the introns of genes in the brain are responsible for the activation of RNA polymerase II transcription from those genes (Sutcliffe, et al., 1984a; 1984b; McKinnon et al., 1986). More recently the capped U6 small nuclear RNA, proposed to be involved in the packaging of hnRNPs was also found to be transcribed by RNA polymerase III (Kunkel et al., 1986; Krol et al., 1987; Reddy et al., 1987; Das et al., 1987). But perhaps the most studied class III genes are 5S RNA genes (Weil et al., 1979; Ng et al., 1979; Bogenhagen et al., 1980; Sakonju et al., 1980; Gottesfeld and Bloomer, 1982; Bogenhagen, 1985; Bieker and Roeder, 1986) and tRNA genes (Sprague et al., 1980; Hofstetter et al., 1981; Ciliberto et al., 1982a; 1982b; Klekamp and Weil, 1982; Folk and Hofstetter, 1983; Schaack et al., 1984; St. Louis and Spiegelman, 1985; Chang et al., 1986; Lofquist and Sharp, 1986; Sajjadi et al., 1987).

Genes transcribed by RNA polymerase III all share certain characteristics. One of the features of class III genes is the presence of a cluster of T residues in the non-coding strand of their 3'-flanking sequences. RNA polymerase III was shown to terminate transcription in 5S RNA and tRNA genes at four or more T residues without the addition of protein factors other than those required for transcription initiation. The absence of the termination signal results in the production of run-on transcripts (Bogenhagen and Brown, 1981; Cozzarelli et al., 1983; Watson et al., 1984; Adeniyi-Jones et al., 1984). One exception

has been found for the termination of transcription in an Alu repeat (Hess et al., 1985) which was shown to terminate at an imperfect hairpin structure formed at the 3' end of the repeat.

The coding sequence of class III genes is capable of promoting transcription. Eukaryotic tRNA genes all contain two highly conserved internal promoter sequences referred to as Box A and Box B (Traboni et al., 1982) or D control and T control regions (Sharp et al., 1981). Alu family and VA RNA genes also contain Box A and B-like sequences (Fowlkes and Shenk, 1980; Ciliberto et al., 1983; Rohan and Ketner, 1987). The 5S RNA coding sequence shares homology and is functionally equivalent to only the Box A of tRNA genes (Ciliberto et al., 1983). The conserved sequences have been shown to bind factors required for the promotion of transcription by RNA polymerase III (Taylor and Segall, 1985; Johnson-Burke and Soll, 1985).

II. Transcription in vitro

Early transcription studies showed that purified RNA polymerase III isolated from Xenopus laevis could not support selective transcription of cloned 5S RNA genes, even though the enzyme maintained polymerizing activity on 5S RNA oocyte chromatin templates (Parker and Roeder, 1977). This suggested the possible requirement of additional transcription factors for activity.

Soluble cell-free extracts to enable faithful RNA polymerase III transcription *in vitro* were first developed for VA RNA using human KB cells (Harris and Roeder, 1978; Weil et al., 1979; Wu, 1980) and HeLa cells (Guilfoyle and Weinmann, 1981). These cell-free extracts were found to also transcribe the Alu family of sequences (Duncan et al., 1979; Jelinek et al., 1980).

Subsequently other cell-free transcription systems from S-100 extracts have been used to faithfully transcribe VA, 5S RNA and tRNA genes. Also, it has been shown that heterologous extracts have decreased stringency in directing tRNA transcription *in vitro* . The stringency varies between different organisms, since certain extracts such as HeLa and *Xenopus* are less selective in directing transcription of *Drosophila* heterologous tRNA genes than are yeast extracts (Schaack et al., 1984; Schaack and Soll, 1985). In addition, most transcription systems studied have shown multiple rounds of transcription initiation.

III. Transcription control regions of 5S and tRNA genes

A promoter is defined as the sequence of DNA required to direct binding of polymerase to initiate transcription. Telford et al. (1979) were able to delimit the promoter region for a $tRNA^{Met}_i$ gene to within 22 bp of the 5'-flank by injection of a series of 5' and 3' deletions of a 120 nucleotide *Xenopus borealis* 5S RNA gene into *Xenopus* oocytes. The aim was to delimit the control regions for

in vitro RNA synthesis (Sakonju et al., 1980). It was found that the 5'-flanking sequences and up to 50 nucleotides of the gene could be deleted before affecting transcription. The 3' boundary for the control region of the 5S RNA gene was found to be at approximately +83 relative to the initiation point of the gene (+1). Thus the control region required for transcription was defined by positions +50 and +83. Also, the position of the control region within the 5S RNA gene along with the immediate 5'-flanking sequences was found to specify the site of initiation, some 50 bp upstream of the internal control region (Sakonju et al., 1980; Bogenhagen et al., 1980).

Though the transcription of tRNA genes was found to have a greater requirement for the 5'-flanking sequences than the 5S RNA genes, two internal conserved sequences (Box A and Box B) were identified within tRNA genes (Ciliberto et al., 1983; Murphy and Baralle, 1984; Stewart et al., 1985). The control regions had previously been shown to be responsible for directing transcription of a *Xenopus laevis* $tRNA^{Met}_{i}$ gene (Hofstetter et al., 1981). Since it was observed that the 5'-flanking sequences of several class III genes revealed very little or no apparent sequence homology, it seemed likely that the internal sequences played a major role in promoter recognition. Therefore the notion that class III promoters were internal became strengthened (Hofstetter et al., 1981).

Ciliberto et al. (1983) have shown that 5S RNA and tRNA genes fall into two different classes. First they showed that the 34 bp internal control region (ICR) of the somatic 5S RNA gene from Xenopus borealis consists of two separate components and can be split by insertion of nucleotides to produce a transcriptionally active maxigene. Second, the first 11 bp of the ICR were shown to be homologous and functionally equivalent to the Box A of a $tRNA^{Pro}$ gene, since hybrid genes constructed from the 5S and tRNA genes were efficiently transcribed in Xenopus laevis oocytes. However, no Box B consensus sequence was found in 5S RNA genes. In addition tRNA and 5S RNA genes initiate transcription at very different distances from their mature coding sequences. Finally, 5S RNA genes bind a specific transcription factor that does not interact with tRNA genes (Sakonju et al., 1981).

The limits of the internal control regions in tRNA genes have been more clearly defined for a Drosophila $tRNA^{Arg}_2$ gene (pArg; Sharp et al., 1981). One promoter element was bound by positions +8 to +25 and was referred to as the D-control or Box A (Sharp et al., 1982). Positions +50 to +58 encode the T-control or Box B internal promoter. In addition, the 5'-half of $tRNA^{Arg}$ genes containing the D-control region plus flanking sequences (i.e. 5' end of the gene) was found to direct RNA synthesis in Kc cell and Xenopus oocyte extracts. Deletion of the 5'-flanking sequences and D-control region abolished transcription in Kc

cell extract, but not in Xenopus oocyte extract (Sharp et al., 1983b). The distance separating the D and T control regions in pArg could be increased between 12 and 77 nucleotides without decreasing the efficiency of transcription or the ability to compete in the binding of transcription factors in Drosophila Kc cell extract (Dingermann et al., 1983). The distance between the D and T control regions is variable among naturally occurring tRNA genes. Some tRNAs contain intervening sequences and it is not unusual to find considerable variation in the lengths of their extra arms (Standring et al., 1981; Baldi et al., 1983).

An experiment increasing the distance between the two control regions of a C. elegans $tRNA^{Pro}$ gene found that a distance of 40 to 50 nucleotides allows for optimal transcription. In addition, a hybrid tRNA gene containing control regions from a $tRNA^{Leu}$ and $tRNA^{Pro}$ genes was found to be transcribed efficiently in Xenopus oocytes, showing that at least in this case the control regions are independent transcription units (Ciliberto et al., 1982a).

A number of studies have focused on the effects of changes in the conserved internal control regions of tRNA genes. Using site-directed in vitro mutagenesis, single and double point mutations were created in the invariant nucleotides of certain tRNA genes which resulted in a decrease in transcription. Such effects further strengthened the notion that promoters of tRNA genes were

internal. Although these residues are implicated in factor binding, there have been inconsistencies in the effects of nucleotide changes in the internal control sequences of different tRNA genes (Stewart et al., 1985; Sharp et al., 1985). Furthermore, it would appear that substituted bases in the internal control region affect transcription differently in different transcription systems (Stewart et al., 1985).

Folk and Hofstetter (1983) carried out an extensive mutagenesis study of the Xenopus $tRNA^{Met}_i$ gene to determine the effects of these mutations on transcription. Transcription was reduced between three to twenty-fold for mutations in the internal control regions of the gene. Mutations in the 3' internal control region of a C. elegans $tRNA^{Pro}$ gene (Goodman et al., 1977) were found to severely affect transcription. An extensive series of mutations were created in the yeast $tRNA^{Tyr}$ gene (Allison et al., 1983). Mutations which reduced template activity were confined to the internal control regions of the $tRNA^{Tyr}$ gene. However, the results showed that mutations at corresponding positions of all these tRNA genes displayed very different effects on transcription.

Nucleotide changes outside the control regions of tRNA genes have also been shown to affect transcription efficiency. For example, mutations in the extra arms of a yeast $tRNA^{Ser}$ gene (Willis et al., 1984), a C. elegans $tRNA^{Pro}$ gene (Traboni et al., 1984) and a yeast $tRNA^{Tyr}$ gene

(Ciampi et al., 1982) all resulted in decreased transcription activities. It has been suggested that the extra arm may represent an extension of the 3' internal control region (Sharp et al., 1985). Mutations in the anticodon stem/loop region between the two internal control regions have also been shown to be important for promoter function (Folk and Hofstetter, 1983). However, mutations in the anticodon stem of the yeast $tRNA^{Tyr}$ gene (Allison et al., 1983) and a yeast $tRNA^{Ser}$ gene (Sharp et al., 1985) did not reduce template activity. In contrast, mutations in the anticodon loop of a Drosophila $tRNA^{Arg}$ gene resulted in a decrease in transcriptional activity (Stewart et al., 1985). The results therefore indicate that sequences outside the internal control regions of tRNA genes are involved in the promotion of transcription and may therefore interact with transcription factors.

The role of Pol III transcription complexes for 5S RNA and tRNA genes has been reviewed by Brown (1984), Lassar et al. (1983) and studied in detail by Andrews et al. (1984) and Lassar et al. (1985). Some of the factors that are required for Pol III transcription have been analyzed by fractionation of crude cellular extracts (Segall et al., 1980; Shastry et al., 1982; Klekamp and Weil, 1982; Johnson-Burke et al., 1983). The first transcription factor purified and shown to bind to the internal control region of 5S RNA genes was a polypeptide of approximately 40 Kd (TFIIIA) (Engelke et al., 1980).

Transcription-competition assays were developed in order to measure the ability of increasing amounts of one gene to inhibit transcription of a second gene. Wormington et al. (1981) found that the 40 Kd protein which is a positive transcription factor acts as a limiting component in the competition assay. Furthermore, deletion of the 5'-flanking region reduced the competitive strength of somatic 5S DNA, but did not affect oocyte 5S DNA in oocyte nuclear extract.

Dingermann et al. (1983) proposed a model in which two transcription factors interact with the D and T control regions respectively and result in template activation by a cooperative mechanism. In this model, the δ factor binds to the D control region and the τ factor to the T control region to bring about stable complex formation. Separation of the two control regions interferes with cooperative stable complex formation as measured by competition experiments. Transcription competition assays using 5' and 3' deletion derivatives of a $tRNA^{Arg}_2$ gene (pArg) showed that the presence of both the D and T control regions is necessary for maximum competitive strength and that both the 5' and 3' flanking sequences contribute to the competitive ability of the D and T control regions respectively (Sharp et al., 1983a; Schaack et al., 1983).

Two transcription factors designated TFIIIB or Factor B(δ) (Mr 260 Kd) and TFIIIC or Factor C (τ) (Mr 300 Kd), are required for the transcription of tRNA genes (Stillman et

al., 1984a; 1984b; 1985; Ruet et al., 1984; Johnson-Burke and Soll, 1985; Camier et al., 1985 Klekamp and Weil, 1986; Marzouki et al., 1986; Klekamp and Weil, 1987). DNA footprint analysis of these factors has shown that while TFIIIC interacts primarily with the T control region, protection can be extended to the D control region as well (Camier et al., 1985; Baker et al., 1986; Carey et al., 1986b). More recently, Yoshinaga et al. (1987) have separated TFIIIC into two functional components TFIIIC1 and TFIIIC2. TFIIIC2 was shown to bind to the T control region and protection was extended to the D control region by the addition of TFIIIC1. An RNA polymerase III factor (TFIIID) isolated from silkworm, has been shown to function in a similar fashion to TFIIIC2 and is probably analogous to TFIIIC2 (Ottonello et al., 1987). Although binding of TFIIIB to the DNA in the absence of TFIIIC has not been demonstrated, its presence is essential for stable complex formation (Lassar et al., 1983; Johnson-Burke and Soll, 1985). In addition to TFIIIB and TFIIIC, 5S RNA genes require a third transcription factor TFIIIA (Gottesfeld and Bloomer, 1982; Hanas et al., 1984; Smith et al., 1984; Bogenhagen et al., 1985) to promote the formation of the stable complex prior to transcription by RNA polymerase III. The binding order of transcription factors in 5S RNA genes is believed to be TFIIIA, TFIIIC and then TFIIIB (Setzer and Brown, 1985). The order of binding of factors in tRNA genes is believed to be TFIIIC followed by TFIIIB, as shown

by transcription competition experiments with both the 5' and 3' control regions of the gene (Lassar et al., 1983; 1985). Interestingly, stable complex formation has been shown to be influenced by the 5'-flanking sequences in both tRNA and 5S RNA genes (Schaack et al., 1984; Johnson-Burke and Soll, 1985; Taylor and Segall, 1985). In addition, deletion of the 5'-flanking sequences of a VA RNAI gene (Fowlkes and Shenk, 1980) was shown to result in reduced competition ability for the factors.

Fractions containing isolated factors have been used in DNase I protection analysis to study the interaction of factors with the gene during complex formation (Fuhrman et al., 1984). For a yeast SUP53 tRNA gene, a mutation in the 3' internal control region was shown to prevent factor interaction and decrease its competitive strength (Newman et al., 1983). However, mutations in the 5' ICR did not affect binding of factors. Also, for a $tRNA^{Tyr}$ SUP4-o gene, stable complexes were formed with the factor alone (Ruet et al., 1984). Interestingly, Wingender et al. (1986; 1987) have shown that complexes of TFIIIB, TFIIIC and RNA polymerase III are capable of forming in the absence of template DNA.

The formation of stable complexes for tRNA genes has been studied by using competitor and reference genes in various assays to test the abilities of various genes in inhibiting the transcription of the reference gene (St. Louis and Spiegelman, 1985). Similar studies have shown the

3' internal control region to be most important in the competition experiments (Sharp et al., 1983a; Schaack et al., 1983). The 5' ICR was also shown to contribute to the formation of stable complexes, since deletion of the 5'-flank reduced competitive ability. Schaack et al. (1983) also showed that while stable complex formation occurred within five minutes for pArg (independent of temperature between 24 and 30° C), transcription was detected after ten to thirty minutes and was in turn temperature dependent. The results therefore showed that two steps were involved in the formation of stable complexes, the second perhaps involving the interaction of another factor or a rearrangement of the formed complex (Sharp et al., 1985).

IV. Modulation of tRNA gene transcription by 5'-flanking sequences

It is well established that 5'-flanking sequences of tRNA genes modulate transcription. The study of tRNA gene transcription revealed a greater requirement for the 5'-flanking sequences than for 5S RNA genes (DeFranco et al., 1980). A cloned Bombyx mori $tRNA^{Ala}_2$ gene lacking all but 11 nucleotides of its 5'-flank was unable to direct efficient transcription in Bombyx extract, but was transcribed in Xenopus extract (Sprague et al., 1980). It was later shown that the 5'-flanking sequences of the $tRNA^{Ala}$ gene between positions -37 and -16 relative to the mature coding sequence were required for efficient

transcription in a homologous extract (Larson et al., 1983; Young et al., 1986). Conserved sequences in the 5'-flank of $tRNA^{Ala}$ genes which were also found upstream of silkworm 5S genes (Morton and Sprague, 1982) were postulated to interact with the RNA polymerase III complex during transcription initiation.

The 5'-flanking sequence requirements have also been shown for a C. elegans $tRNA^{Pro}$ gene (Ciliberto et al., 1982a) and a yeast $tRNA^{Tyr}$ gene (Shaw and Olson, 1984). In fact, it has been shown that the 5'-flanking region of a yeast $tRNA^{Leu}_3$ gene is sufficient to constitute an RNA polymerase III promoter with either the A or B Box sequences in homologous cell-free extracts (Johnson and Raymond, 1984). A deletion of the 5'-flanking sequence of this yeast $tRNA^{Leu}_3$ gene to position -2 was still active in Xenopus extracts, but nearly inert in a homologous extract (Raymond and Johnson, 1983; Johnson and Raymond, 1984).

The 5'-flanking sequences of tRNA genes studied to date are characterized by the lack of conserved nucleotide sequences. These differences between the 5'-flanks may reflect the specificities of various RNA polymerase III enzyme complexes for different control elements in different organisms. This has best been demonstrated by the observation of very different transcription efficiencies for tRNA genes transcribed in heterologous extracts (Sharp et al., 1982; Schaack et al., 1984; Schaack and Soll, 1985; Glew et al., 1986). The differences may suggest that some

component(s) in the heterologous extract does not bind in the same manner as the homologous component(s) to the promoter (Johnson-Burke and Soll, 1985).

The exchange of 5'-flanking sequences between different tRNA genes has shown that a specificity is required between the gene and its 5'-flanking region. This has been demonstrated for a $tRNA^{His}$ pseudogene of Drosophila (Cooley et al., 1984). The $tRNA^{His}$ pseudogene was a poor template for transcription in homologous extracts, whereas its counterpart, a $tRNA^{His}$ gene, which possesses different flanking sequences, was very efficient in directing transcription. When the 5'-flank of the pseudogene was replaced with that of the wild type $tRNA^{His}$ gene, transcription activity was restored. However, replacement of the 5'-flank of the $tRNA^{His}$ pseudogene with the 5'-flanking sequences of the active pArg gene, did not restore transcriptional activity. In addition, while HeLa cell extract has been used to transcribe efficiently all tRNA genes examined to date, this extract failed to direct efficient transcription of either $tRNA^{His}$ genes (Cooley et al., 1984).

A termination-like sequence as part of an undecanucleotide was found to be responsible for reduced template activity in Drosophila $tRNA^{Lys}_2$ genes when present in the 5'-flanking region. The inhibitory effects of this sequence were reduced by its positioning away from the mature coding sequence and inhibition by the sequence was

removed by its deletion from the 5'-flank (DeFranco et al., 1981). Similar sequences have been found in the 5'-flanking region of a Drosophila $tRNA^{Arg}$ gene (Dingermann et al., 1982), a Drosophila $tRNA^{Leu}$ gene (Glew et al., 1986) and a Drosophila $tRNA^{Val}_4$ gene which is the subject of this study. A deletion series in the 5'-flank of the $tRNA^{Val}_4$ gene (pV4a.5-179) delimited a negative modulatory sequence consisting of thymidylate tracts flanked by purines between positions -70 and -50 relative to the mature coding sequence (Sajjadi et al., 1987). The only other inhibitory sequence found has been a 12 bp alternating purine/pyrimidine sequence in the 5'-flanking region of two $tRNA^{Met}$ genes from Xenopus laevis (Hipskind and Clarkson, 1983).

The most extensive deletion studies of the 5'-flanking sequences of RNA polymerase III genes have been for a Drosophila $tRNA^{Arg}_2$ gene by Schaack et al. (1984) and a Drosophila $tRNA^{Val}_4$ gene by Sajjadi et al. (1987). For pArg, deletion of the 5'-flanking sequences to position -36 resulted in a higher level of transcription than for pArg. The increased efficiency of transcription was abolished by deletion to position -33 and transcription was only 12% of pArg for deletion -32 (Schaack et al., 1985). Further deletion of the 5'-flank to position -17 resulted in a gradual loss in transcription efficiency, with deletion -11 transcribing at less than 1% of pArg. As a result, the 5'-flanking modulatory sequences of pArg were delimited to

position -60, -33 and -11 (Schaack et al., 1984), but no conserved positive modulatory sequences were identified.

One possible positive modulatory sequence has been delimited for a human $tRNA^{Glu}$ gene that is very efficient in directing transcription (Goddard et al., 1983). This gene contained a sequence with the potential to form a tRNA-like structure in its 5'-flank. Until recently tRNA genes from higher eukaryotes were thought not to be modulated transcriptionally by their 5'-flanks (Schaack and Soll, 1985). Arnold et al. (1986; 1987) have shown that different 5'-flanking sequences of human $tRNA^{Val}$ genes contain extragenic control sequences responsible for effecting transcription efficiency. In addition, the 5'-flanking regions of mouse $tRNA^{His}$ genes have also been shown to modulate their transcription (Morry and Harding, 1986). While a conserved sequence in the 5'-flank of a number of Drosophila 5S and tRNA genes has been identified, the effects of this sequence on the transcription of those genes have not been examined (Indik and Tartof, 1982).

For a Drosophila $tRNA^{Val}_4$ gene (Sajjadi et al., 1985; 1987), deletion of 5'-flanking sequences to position -49 resulted in a 44% increase in transcription relative to the wild type template. Further deletions to position -39 gradually lowered the Vmax and deletion to position -37 resulted in a sharp drop in transcription efficiency. Additional deletions of the 5'-flank severely reduced the level of transcription. These results delimited a sequence

of the general form TNNCT where N is any nucleotide. TNNCT was also found associated with efficient transcription of other Drosophila tRNA genes.

V. Present investigations

The deletion of 5'-flanking sequences in pV4a.5-179 (Sajjadi et al., 1985; 1987) suggested the sequence TNNCT was a positive modulator of tRNA gene transcription. In addition, the effects observed indicated that for certain deletion end-points, a TNNCT present in nearby vector sequences was responsible for influencing $tRNA^{Val}_4$ transcription. Therefore to determine the possible modulatory effects of TNNCT in the absence of vector sequences, a number of site-specific changes have been created in the sequence TNNCT between positions -33 and -38 of pV4a.5-138, a template which directs transcription at wild type levels. In addition, a number of transcription experiments were carried out to determine the function of TNNCT in directing transcription. These studies were extended to experiments in which temperature and ionic strength were altered to determine the role of TNNCT in stable complex formation. The results showed that the pentanucleotide was not involved in determining the efficiency of formation or the maintenance of complexes on the gene but is probably responsible for affecting the rate of transcription initiation. In an additional study a series of deletions extending into the 5'-flank of a

Drosophila $tRNA^{Ser}_{7}$ gene lacking a TNNCT sequence were created. Results obtained from transcription of deletion end-points delimited a second possible positive modulatory sequence which was also present in the $tRNA^{Val}_{4}$ gene.

MATERIALS AND METHODS

I. Site-specific mutagenesis

A. Preparation of dU containing single-stranded template

A loop of glycerol culture from a stock of *E. coli* strain BW313 (Hfr KL 16 PO/45 [*lys A* (61-62)], *dut*1, *ung*1, *thi*1, *rel*A1; Kunkel et al., 1987) was used to inoculate 2 mls of 2YT (16 g yeast extract; 10 g bacto tryptone; 10 g NaCl). The culture was grown at 37° C on a fast roller to A_{660}=0.8, and streaked for single colonies on a 2YT agar plate, incubating at 37° C overnight. It is important to prepare the bacteria in this manner, otherwise the single-stranded template will have a low yield and will be contaminated with a high amount of helper phage DNA, chromosomal DNA and RNA.

A single BW313 colony was picked and used to inoculate 2 mls of 2YT. The culture was grown as described above to A660= 0.2. Of this culture 200 ul was transferred to 2 mls of 2YT containing 1 ul of the appropriate phage supernatant of the desired clone (either pTZ [USB] or pEMBL8- [Dente et al., 1983] at 10^{11} pfu/ml) diluted 10^2-10^3 times (see section A.4). Cells were placed at 37° C for 30 min and 100 ul of the culture was plated on a 2YT plate supplemented with 70 ug/ml ampicillin. Colonies were grown overnight at 37° C.

A single transfected colony was grown in 2 mls of 2YT/ampicillin as described above to A_{660}= 0.5. The culture

was superinfected with 40 ul of M13K07 helper phage ($2x10^{11}$ pfu/ml; Pharmacia) for 1 hr at 37° C and then transferred to 50 mls of 2YT supplemented with 70 ug/ml kanamycin (Boehringer Mannheim) and 0.25 ug/ml uridine (Sigma). Cells were grown for 18 hrs and separated from the supernatant by two consecutive centrifugations at 6000xg for 15 min in a SS34 rotor. The phage were collected by 1) precipitation with the addition of 1/4 volume of 20% PEG in 3.5 M NH_4OAc (Fisher) and incubation on ice for 30 min, and 2) centrifugation at 6000xg for 15 min. The phage pellets were suspended in 200 ul of dH_2O and extracted at least three times with an equal volume of phenol/chloroform (24:24:1, phenol:chloroform:isoamyl alcohol) and finally with an equal volume of chloroform. The solution was made 3 M by the addition of 7.5 M NH_4OAc (BDH) and the DNA was precipitated by the addition of 2.5 volumes of 95% ethanol. The DNA was collected by centrifugation and the pellet was then suspended in 0.25 M NaOAc (BDH) and precipitated with 95% ethanol. The pellet was washed with 1 ml of 80% ethanol, dried under vacuum and suspended in 50 ul of dH20. 5 ul was electrophoresed on a 0.7% agarose gel to estimate yield and purity of single-stranded plasmid DNA.

M13K07 was prepared from areas of densely packed single plaques on a 2YT/kanamycin plate which were used to inoculate 50 mls of 2YT/kanamycin as described above, except that the phage were not precipitated.

B. Testing pEMBL and pTZ for dU content

2 mls of 2YT were inoculated with E. coli BW313 and NM522 (hsd Δ5, Δ(lac-pro), [F',lac I^qZ ΔM15,pro^+]) separately and grown to an A_{660}= 0.2 at 37° C. The cells were infected with 1 ul (~10^{11} pfu/ml) of phage supernatant from A1. After 30 min, 100 ul of cells from each infection was plated on 2YT plates containing 70 ug/ml ampicillin. Plates were incubated at 37° C overnight. On the average only a few colonies of BW313 appeared on the plate while there were over one hundred thousand colonies of NM522.

C. Purification of deoxyoligonucleotides

The following oligonucleotides were synthesized on an Applied Biosystems Model 380-A DNA synthesizer by T. Atkinson (University of British Columbia): 5'-CGTTGAGGGCGCTGAAG-3'; 5'-CAGTTGAGG(GA)CG(CAG)(TAG)GAAG-3'; 5'-GTTGAGGTCGATGAAGTTGGC-3'; 5'-GTTGAGGTCGCAGAAGTTGGCC-3'; 5'-GTTGAGGGCGATGAAGTTG-3'; 5'-GTTGAGGGCGGTGAAGTTG-3'; 5'-GCAGTTGAGGTTAGCTGAAGTTG-3'; 5'-GCAGTTGAGGTCTCTGAAGTTG-3'; 5'-GCGGTTATCACATCAGCGCAACACGCAGAAGG-3'. Nucleotides in brackets indicate mixed oligonucleotide sequence. Oligonucleotides were separated by electrophoresis on 16% or 20% polyacrylamide gels containing 7 M urea, the DNA was detected by U.V. shadowing of a fluorescent T.L.C. plate and the least mobile band was excised from the gel. Gel fragments were crushed, covered with 0.5 M NH_4OAc in 1.5 ml Eppendorf tubes and incubated overnight at 37°. The eluate was cleared of gel fragments by passage through a 45 u

Millipore disc (Millex HV4) and the DNA purified on a reverse phase C18 SEP-PAK cartridge (Waters) as previously described (Atkinson and Smith, 1984).

D. Mutagenesis

Deoxyribonucleotides were phosphorylated and used in the two-primer mutagenesis procedure as previously described (Zoller and Smith, 1984) except that 10 pmoles of phosphorylated oligonucleotide was used and the primer extended at 16° C for 45 min followed by a second 45 min incubation at 25° C. Forward and reverse sequencing primers (Pharmacia) were used for pTZ19U and pEMBL8- plasmids respectively. Reaction mixtures were diluted five-fold in 50 mM $CaCl_2$/10 mM Tris-Cl (pH=8.0) and used to transform NM522 prepared as described by Maniatis et al. (1982). Cells were plated on 2YT plates containing 70 ug/ml ampicillin and incubated at 37° C overnight. Colonies were used to inoculate 2 mls of 2YT/ampicillin as described in A.1. Following superinfection with M13K07, 120 ul of culture were transferred to 3 mls of 2YT/kanamycin and grown for 18 hrs. Single-stranded plasmid was prepared as in A.1. except that centrifugations were for 2 mls of culture in a microfuge (Eppendorf). Prior to phenol/chloroform extraction, phage pellets were suspended in 100 ul of 50 mM Tris-Cl (pH=7.5), 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and treated with 100 ug of RNase A (Sigma) for 30 min at 37° C. After deproteinization, the DNA was precipitated twice with ethanol and suspended in 12 ul of dH_2O. Four ul were

analyzed by electrophoresis on a 0.7% agarose gel to assess DNA yield and purity.

II. Chain-terminator sequencing

All dideoxy and deoxyribonucleoside triphosphates (Pharmacia) were adjusted to pH 7.0 with Tris base and their concentrations determined by spectrophotometry at the appropriate wavelengths as described by Maniatis et al. (1982). All dideoxynucleotide mixes were prepared as described by Messing et al. (1981). Stocks of dideoxynucleotides (5 mM) and deoxynucleotides (10 mM) were aliquoted, maintained at -20° C and thawed only once.

Single-stranded DNA (1-2 ug) was used for dideoxy sequencing as described by Sanger et al. (1977) using both the forward (pTZ19U) and reverse (pEMBL8-) primers supplied in solution (Pharmacia). Reactions contained 7 ul of DNA in dH_2O, 1 ul of 10X Hin buffer (66 mM Tris.Cl [pH 7.4], 66 mM $MgCl_2$, 165 mM NaCl and 66 mM BME) and 2 ul of the appropriate primer solution. The mixtures were incubated at 55° C for 5 min and an additional 5 min at room temperature, at which time 1 ul of 0.1 M DTT, 1 ul of 15 uM dATP, 1 ul (10 uCi) of [α-^{32}P] dATP, NEN, 3.16x10^3 Ci/mmole) and 1 ul(3 units) of Klenow DNA polymerase (BRL) were added. Three ul of the mixture was aliquoted into four tubes containing 2 ul of the appropriate dideoxynucleoside triphosphate /deoxynucleoside triphosphate mix. Reactions were carried out at 30° C. After incubation for 15 min, 1 ul of cold

chase nucleotide mix containing 0.3 mM dNTPs and 0.3 units of Klenow polymerase was added to each of the four reaction tubes and incubation continued for an additional 15 min. Reactions were terminated by the addition of 7 ul of stop mix (90% formamide [BRL], 25 mM EDTA and 0.01% xylene cyanol and bromophenol blue) and heated at 90° C for 3 min prior to electrophoresis.

III. Sequencing by chemical degradation

Twenty ug of the $tRNA^{Val}_4$ gene pV4a.5-45, cloned in the EcoRI/HindIII site of pEMBL8- were treated with AvaI (40 units,New England Biolabs). One ug was subjected to electrophoresis on a 0.7% agarose mini-gel to ensure linearization of the DNA fragment. The remaining DNA was end-labelled using the Klenow polymerase (15 units,BRL) with 50 uCi of [α-^{32}P] dCTP (600 Ci/mmol,NEN) in the same buffer, as described by Maniatis et al. (1982). The DNA was precipitated with 95% ethanol, digested with EcoRI (40 units,Pharmacia) and analyzed directly on a native 5% polyacrylamide gel by electrophoresis. The gel was subjected to autoradiography for 3 min and the 106 bp EcoRI/AvaI fragment was excised from the gel. DNA was recovered from the gel pieces by soaking in 0.5 M NH_4OAc/10 mM MgOAc at 50° C overnight. The eluate was extracted with an equal volume of phenol/chloroform and precipitated with 95% ethanol. DNA was reprecipitated with ethanol in the presence of 0.25 M NaOAc, washed with 1 ml of 80% ethanol

and desiccated under vacuum. DNA was suspended in 50 ul of dH_2O and 10 ul ($1.4x10^6$ cpm) was subjected to chemical degradation sequencing as described by Maxam and Gilbert (1980). Cleavage products were resolved on an 8% polyacrylamide/ 7 M urea gel and subjected to autoradiography.

IV. Gel-retardation assay

Twenty five ug of DNA containing the Val_4 5'-flank cloned in the HindIII site of pEMBL8- was digested with HindIII (90 units, Pharmacia) and end-labelled in the same buffer with 15 units of Klenow polymerase and 100 uCi (3000 Ci/mmol) [α-^{32}P] dATP as described by Maniatis et al. (1982). Products were analyzed by electrophoresis on a native 5% polyacrylamide gel and subjected to autoradiography for 15 seconds. The 185 bp fragment was excised from the gel, covered with 0.2 M NaCl in Tris-EDTA (T.E., 10mM Tris-Cl [pH=8.0], 1 mM EDTA) and eluted at 55° C overnight. The eluate was run on a NACS PREPAC minicolumn as described by the NACS manual (BRL) and the DNA precipitated with ethanol. DNA was washed with 80% ethanol, desiccated under vacuum and suspended in 20 ul of T.E. Binding reactions (30 mM Tris-Cl[pH=7.9], 120 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT) contained 30,000 cpm (~0.5 ng) of labelled probe DNA, 1.5 ug of pUC13 or 3 ug of poly dI-dC (Pharmacia) as non-specific DNA and between 0.05-0.9 ul of S-100 extract in a total volume of 20 ul. Reactions were composed on ice and following a 10 min

incubation at 23.5° C, labelled DNA was added and incubation continued for 45 min. Two ul of running buffer (50% glycerol, 2XTGE, 0.1% bromophenol blue and xylene cyanol) were added to each and samples were applied to a 4% native polyacrylamide gel. Samples were electrophoresed in TGE (50 mM Tris-Cl[pH=8.5], 380 mM glycine, 2 mM EDTA) as described by Singh et al. (1986). The gel was transferred onto Whatman 3MM paper, dried and subjected to autoradiography overnight.

V. In vitro transcription and analysis of RNA products

Transcription reactions (50 ul) contained 19 mM Tris-Cl [pH=7.9], 110 mM KCl, 7 mM $MgCl_2$, 3mM DTT, 2.5 ug/ml α-amanitin (Boehringer Mannheim), 6.5 units/ml creatine phosphokinase, 5 mM phosphocreatine, 6 mM ATP, GTP, CTP, 25 uM [α-^{32}P] UTP (410 Ci/mmol), 5 to 100 ng of template DNA (unless otherwise indicated), up to 1 ug of pUC8 or 13 DNA and 25 ul of *Drosophila* Schneider II S-100 cell extract, as described by Rajput et al. (1982). Reactions were carried out at 23.5° C for 90 min (unless otherwise specified) and terminated by the addition of 60 ul stop mix (170 ug/ml tRNA [Pharmacia], 0.5% SDS, 50 mM NaOAc) and 90 ul of dH_2O. The mixture was extracted with 200 ul of phenol and the aqueous phase precipitated with two volumes of 95% ethanol and 10 ul of 0.04 M ATP.

The pellet was dried under vacuum, suspended in 5 ul of T.E. (pH=7.5), 9 ul running dye (10 M urea, 20 mM Tris-Cl

[pH=7.5], 1 mM EDTA, 0.1% bromophenol blue and xylene cyanol) and heated at 90° C for 3 min. Products were analyzed by electrophoresis on 8%, 10% or 15% polyacrylamide gels containing 7 M urea. Following autoradiography, gel slices containing radioactive tRNA bands were excised from the gel and counted for Cerenkov radiation in order to quantitate the amount of product.

VI. Calculations and statistical analysis

All transcriptions were the result of two different S-100 extracts (unless otherwise specified). Control transcriptions were included in every experimental run. Values for Vmax were derived from the calculation and plot of a linear regression of a Lineweaver-Burke plot (1/V vs. 1/S) and expressed relative to Vmax for the control for that experiment. Vmax values expressed as % increase or decrease relative to the control for each template derived from experiments with separate extracts were reported as the average between the two extracts.

VII. Large-scale isolation of plasmid DNA

Either a loop of glycerol culture or a loop of cells from a plate containing the pUC or pEMBL plasmid clones was used to inoculate 25 mls of 2YT supplemented with 70 ug/ml ampicillin (2YT/amp) in a 100 ml flask and shaken at 37° C until the culture reached late log phase (A_{660}=0.7); all of this culture was used to inoculate 500 ml of 2 YT/amp in a 2 L flask which was incubated at 37° C with vigorous shaking

until late log was reached, at which point 2.5 ml of chloramphenicol (34 mg/ml in ethanol) was added (final concentration was 170 ug/ml). Incubation was continued with vigorous shaking for a further 18 hrs.

The alkali lysis procedure described by Maniatis et al. (1982) was used to isolate DNA, with the following modifications. Cells were harvested in a Sorval GSA rotor at 10,000xg, 4° C, for 12 min. Treatment with lysozyme and subsequent lysis was carried out as described, except that the cells were maintained in 250 ml plastic bottles. The lysate was centrifuged in a Sorval GSA rotor at 4° C, 13,000xg for 20 min and the cleared lysate transferred to two 50 ml polycarbonate tubes. Following precipitation with isopropanol, the DNA was recovered by centrifugation at 12,000xg in a Sorval SS34 rotor for 30 min at 23° C. The nucleic acid pellet was then washed with 5 ml of 70% ethanol and following brief desiccation was dissolved in 5.5 ml of T.E. (pH=8.0). CsCl (6.5 g) was added and dissolved by gentle agitation, followed by the addition of 0.7 ml ethidium bromide (2 mg/ml in dH_2O). The insoluble material was removed by a 5 min centrifugation in a Sorval SS34 rotor at 6,000xg. The solution of DNA and CsCl was transferred to 13.5 ml polyallomer tubes and centrifuged to equilibrium in a Beckman 70.1 Ti rotor at 109,000xg, at 8° C for 40 hrs. The supercoiled plasmid DNA band was removed, extracted with dH_2O-saturated n-butanol and dialyzed against three lots of 4 L of T.E. (pH=7.5) at 4° C for 2 hrs. Following the

determination of plasmid concentration at OD_{260} (1 OD_{260}=50 ug/ml of DNA), 1 ug of DNA was run on a 0.7% agarose gel to assess the degree of superhelicity.

VIII. Small-scale isolation of recombinant DNA

The alkali-lysis method, essentially as described by Maniatis et al. (1982), was used to isolate recombinant plasmid DNA from 2 ml cultures. Following extraction with phenol/chloroform, the DNA was extracted with 1/2 volume of chloroform and 1/2 volume of 7.5 M NH_4OAc was added and the DNA stored on ice for 20 min. The solution was centrifuged in a microfuge (Eppendorf) for 15 min and the pellet discarded. The supernatant was precipitated with two volumes of 95% ethanol, washed with 1 ml 80% ethanol and desiccated under vacuum. The DNA pellet was then suspended in an appropriate volume of the desired restriction buffer for further analysis. DNA was treated with RNase A (40 ug/ml) at the same time as digestion with restriction enzymes.

IX. Digestion of DNA with restriction endonucleases

Restriction endonucleases were obtained from New England Biolabs and Pharmacia and were used in accordance with the manufacturer's specifications. In general, 10 units of restriction enzyme per ug of DNA were used and incubation was for 2 hrs at 37° C (1 unit of enzyme activity

is the amount of enzyme required to completely digest 1 ug of lambda DNA in 60 min in a total of 50 ul at 37° C.

X. Agarose gel electrophoresis

All native gels contained TBE buffer (0.089 M Tris-base, 0.089 M Boric Acid, 0.002 M EDTA); the sample running dye contained 50% glycerol, 2xTBE, 0.1% bromophenol blue and xylene cyanol. Either 0.6%, 0.7%, 0.8% or 1% agarose gels (Biorad) were used to analyze supercoiled or restriction endonuclease treated plasmid DNA as well as single-stranded DNA. Mini-gels were cast on 5 cm x 7.5 cm glass slides and contained 0.001% ethidium bromide (EtBr). The gels were subjected to electrophoresis at 60 mA, with constant current.

XI. Polyacrylamide gel electrophoresis

Native polyacrylamide gels (17 cm x 23 cm or 14 cm x 18 cm), either 4%, 5%, 6%, 7% or 8%, were prepared from a stock solution of 45% polyacrylamide (43.5% acrylamide:1.5% N,N'-methylene-bis-acrylamide). TBE buffer (as above), 0.05% ammonium persulfate (w/v) and 0.1% (v/v) TEMED (Biorad) were used in all these gels. DNA samples were electrophoresed at 200 volts, constant voltage and the gel was stained in 0.1% EtBr in TBE for 15 min prior to viewing with a UV transilluminator.

Denaturing polyacrylamide gels (8%, 10% or 15% polyacrylamide containing 7 M urea) were used for analysis

of transcription products. Conditions for gel preparation were as described above except that the TBE buffer contained 0.05 M Tris-base, 0.05 M boric acid and 0.001 M EDTA. Products were run at 600 volts, constant voltage for 1.5 hrs and tRNA bands detected by autoradiography at -70° C.

For DNA sequencing gels (20 cm x 44 cm), 3 ul of denatured samples were loaded onto the wells of either 6%, 8% or 10% gels containing 7 M urea. Conditions for the preparation of gels were as described for transcription gels, except that the running dye contained 90% formamide (BRL), 25 mM EDTA and 0.1% bromophenol blue and xylene cyanol; gels were run at 1700 volts, at constant voltage.

Native polyacrylamide, denaturing polyacrylamide and DNA sequencing gels were 1.5 mm, 0.75 mm and 0.4 mm in thickness, respectively.

XII. Isolation of DNA from agarose gels

Restriction fragments were recovered from 0.6%, 0.7% or 0.8% agarose mini-gels by electrophoresis of the desired band onto an ion-exchange DEAE-nitrocellulose membrane (NA45, Schleicher and Schuell), which had been placed in front of the leading edge of the band. NA45 membranes were activated as described in the Schleicher and Schuell manual. The membrane was placed in a 1.5 ml Eppendorf tube, covered with 500 ul of 1 M NaCl in T.E. (pH=7.5) and the DNA eluted from the membrane at 55° C for 30 min. The solution was extracted once with dH_2O-washed n-butanol to remove EtBr and

precipitated with two volumes of 95% ethanol. The sample was washed with 1 ml 70% ethanol and dried under vacuum. DNA was suspended in dH_2O and an aliquot electrophoresed on an agarose mini-gel to assess the concentration of recovered DNA.

XIII. Isolation of DNA from polyacrylamide gels

Following digestion and electrophoresis of DNA fragments, polyacrylamide gels were stained by EtBr and gel slices containing the desired DNA fragment were excised from the gel. Gel slices were cut up and placed into 1.5 ml Eppendorf tubes and covered with 0.2 M NaCl in T.E. (pH=7.2). The tubes were then placed at 50° C for 16 hrs and the eluate containing the desired DNA fragment was removed and purified on NACS PREPAC (BRL) ion-exchange mini-columns. Procedures for binding, elution and precipitation of DNA were as described in the NACS Applications Manual (BRL).

XIV. Treatment of DNA with exonuclease BAL-31

To create a series of deletions in the 5'-flanking sequences of the $tRNA^{Ser}_7$ gene, a 750 bp EcoRI/HindIII fragment containing the gene, 5' and 3'-flanking sequences was isolated from the pEMBL8- vector and resolved on a 4% polyacrylamide gel. The DNA was eluted and purified on a NACS mini-column. An aliquot of the fragment was analyzed

by electrophoresis on a 0.7% agarose mini-gel to assess recovery and yield.

Approximately 8 ug of the 750 bp fragment was in turn digested with HhaI (80 units) and the products resolved on a 5% polyacrylamide gel. The 391 bp fragment containing the gene, 125 bp of 5'-flanking sequence and 185 bp of 3'-flanking sequence was excised from the gel, the DNA eluted and purified on a NACS mini-column. An aliquot of the fragment was analyzed by electrophoresis on a 1% agarose mini-gel to assess recovery and yield.

Approximately 1 ug of the 391 bp fragment was treated with 1 unit of BAL-31 in a total volume of 50 ul at 30° C in each of three separate tubes. Reactions were terminated after 0.5, 1.0 and 1.5 min by the addition of 50 ul of dH_2O and 100 ul of phenol/chloroform. The DNA was precipitated with 95% ethanol in the presence of 2.5 M NH_4OAc and the pellet washed with 1 ml of 80% ethanol. The dried pellets were suspended in 20 ul of dH_2O and labelled with Klenow polymerase in the presence of unlabelled dCTP, dGTP, TTP and 10 uCi of [α-^{32}P] dATP (see section XV). Reactions were analyzed by electrophoresis on a 5% native polyacrylamide gel with the appropriate DNA size control (i.e. the same 391 bp fragment labelled, but not treated with exonuclease) and subjected to autoradiography for 3 hrs. Areas of the gel corresponding to fragments ranging in size between 390 and 260 bp were eluted from the gel (see section M) and purified on a NACS mini-column. Fragments were subsequently cloned

into the SmaI site of pEMBL8- and used to transform E. coli NM522. Deletion end-points recovered from this BAL-31 series contained at least 117 bp of 5'-flanking undeleted DNA.

In order to create a more extensive series of deletions in the 5'-flank of the $tRNA^{Ser}_{7}$ gene, a deletion clone containing 119 bp of 5'-flanking sequence and 184 bp of 3'-flanking DNA was digested with EcoRI and HindIII and the insert purified from a 5% polyacrylamide gel as described above. Approximately 4 ug of insert DNA were treated with 2 units of BAL-31 in a total volume of 40 ul at 30° C. Aliquots were removed from the reaction at 0.5, 1.0, 1.5 and 2.0 min and reactions terminated as previously described. All further manipulations were as outlined above.

XV. End-labelling and fill-in reactions of DNA

The Klenow fragment of E. coli DNA polymerase was used to label and fill-in 5'extended ends generated by digestion of restriction endonucleases or exonuclease BAL-31. Reactions were carried out in a total of 30 ul at 25° C in Hin buffer and contained 1.5 ul of the appropriate dNTPs (2 mM stock) and 1-3 ul (10-30 uCi) of [α-^{32}P] dATP or [α-^{32}P] dCTP as well as 3 units of Klenow (BRL) enzyme. The unlabelled dNTP was omitted from the reaction containing the labelled dNTP. Reactions were carried out for 20 min at which time 1.5 ul of the remaining absent unlabelled dNTPs

were added and the reaction continued for a further 5 min before termination.

XVI. Autoradiography

Gels containing radioactively labelled DNA or RNA were covered with a layer of plastic (Saran Wrap) and subjected to autoradiography using 3M HiLite X-Ray film. Native and denaturing gels were placed at 4° C and -70° C respectively.

DNA sequencing gels and gels containing bound proteins were dried onto Whatman 3MM chromatography paper using a Biorad Slab Gel drier Model 1125B prior to autoradiography at room temperature. Exposure times for transcription and sequencing gels were for 14-18 hrs and the X-Ray film was developed according to the manufacturer's specifications.

XVII. Ligation of DNA fragments

A. BAL-31 deletion series

The vector pEMBL8- (Dente et al., 1983) (5 ug in 30 ul) was digested with 20 units of SmaI for 2 hrs at 37° C and the DNA purified on a NACS column. An aliquot was electrophoresed on a 0.7% agarose gel to assess linearization and DNA yield. Vector DNA was suspended in dH_2O to a final concentration of 0.1 ug/ul and stored at -20° C. The amount of labelled insert DNA recovered following purification on NACS had to be approximated.

Ligation reactions were carried out in a total volume of 15 ul containing 50 mM Tris-Cl (pH=7.8), 10 mM $MgCl_2$, 15

mM DTT, 0.5 mM ATP and 50 ug/ml BSA. The total amount of DNA in the reaction was ~0.2 ug at a 2:1 molar ratio of vector to insert. Four units of T_4 DNA ligase (BRL) and 0.2 units of T_4 RNA ligase (Pharmacia) were added to each ligation reaction and reactions were incubated at 16° C for 16 hrs.

B. pV4a.5-45

The Val_4 deletion end-point pV4a.5-45 (185 bp) had been previously cloned in the SmaI site of pUC8 along with two similar-sized fragments of pBR322 (present at the HindIII site 3' to the gene; Sajjadi, 1985). To liberate the pBR322 sequences, 8 ug of this construct were digested with 80 units of HindIII at 37° C for 2 hrs and electrophoresed on a 0.6% agarose gel. The largest fragment containing the pUC8 vector and the Val_4 insert was isolated and purified from the gel as described in section XII except that the DNA was further purified on a NACS column. An aliquot was analyzed by electrophoresis on a 0.6% agarose gel to assess the amount of DNA recovered. The DNA was recircularized at the HindIII site by ligation as described in section XVII.A, except that T_4 RNA ligase was omitted from the reaction.

To clone the 185 bp pV4a.5-45 fragment in pEMBL8-, 20 ug of the pUC8 vector harboring the deletion were digested with 200 units of EcoRI and HindIII. The products were applied directly to a 6% polyacrylamide gel and separated by electrophoresis. The liberated fragment was excised and purified as described in section XIII. Vector DNA was

prepared by digestion of 8 ug of pEMBL8- with EcoRI and HindIII and electrophoresis on a 0.6% agarose gel to liberate the multiple cloning site. The vector was excised from the gel and purified as described in section XII. Aliquots of purified insert and vector were analyzed by electrophoresis on a 1% agarose gel to estimate DNA yield. Ligation reactions were as described above.

C. $\underline{tRNA^{Val}_{4}\ 5'\text{-flank}}$

A construct (gift of D. Horvath) in the vector pUC8 was used as the source for the Val_4 5'-flank (179 bp plus 2 nucleotides of the structural gene) which had been isolated and cloned using HindIII linkers. Since the sequences at the 3' end of the 5'-flank had not been characterized, the 5'-flank was recloned in a separate vector. 5 ug of pEMBL8- were digested with HindIII and purified as described in XVII.A. 15 ug of the pUC8 construct were digested with HindIII and the 5'-flank resolved by electrophoresis on a 5% polyacrylamide gel. The insert was excised and purified on a NACS column as previously described. Aliquots of vector and insert were electrophoresed on a 1% agarose gel prior to ligation as described in XVII.B.

D. $\underline{pV4a.5\text{-}138}$

pEMBL8- was digested with EcoRI and HindIII and purified as described in XVII.B. The Val_4 deletion endpoint $\triangle$5'-138 (276 bp) had previously been cloned in the SmaI site of pUC8 (Sajjadi, 1985) along with pBR322 sequences. The pUC8 clone containing this construct (30 ug)

was digested with 100 units of EcoRI and HindIII at 37° C for 2 hrs and analyzed on an 8% polyacrylamide gel by electrophoresis to separate the insert from pUC8 and the pBR322 fragment (338 bp). The 276 bp fragment was excised and purified on a NACS column. Vector and insert were ligated as described in XVII.B.

The pV4a.5-138 cloned in pTZ19U was constructed in similar fashion. Vector pTZ19U (4 ug, USB) was digested with EcoRI and HindIII and purified as described in XVII.B. Deletion end-point pV4a.5-138 was used as insert in cloning as outline above.

Single and double point mutants in the 5'-flank of Val_4 which had been created in pTZ19U were subcloned back into the EcoRI/HindIII site of pEMBL8- as described above.

XVIII. Transformation of E. coli strains JM83 and NM522

The procedure described by Maniatis et al. (1982) was used, except that cells were grown in 2YT medium and only 1/4 the volume of cells (25 ml) was treated with $CaCl_2$/Tris-Cl (pH=8.0). All subsequent volumes were adjusted to account for the decreased number of cells. Either 1 hr or 16 hr $CaCl_2$-treated competent cells were transformed with the entire volume of the ligation mix which had been diluted five-fold. Cells were heat-shocked and following the addition of 1 ml 2YT media, were incubated at 37° C for 1 hr to allow expression of ampicillin resistance before plating.

Following incubation, 90 ul of the transformant cell suspension was plated on 2YT/amp plates with 50 ul of 2% X-gal (Sigma) (plus 10 ul of 100 mM IPTG for NM522 cells only) and incubated overnight at 37° C.

White colonies carrying recombinant plasmids were picked and grown in 2 ml of 2YT/amp media at 37° C with vigorous aeration for plasmid screening. Recombinants were stored at -70° C in vials containing 4 ml of 2YT/amp, 20% glycerol (v/v).

XIX. Screening of recombinant clones

White colonies containing recombinant pUC8, pEMBL8- or pTZ19U clones were used to inoculate 2 ml 2YT/amp for small-scale plasmid preparation as outlined in section H. Plasmid DNA was suspended in 20 ul of dH_2O. One half the amount was used for restriction analysis and the remainder was stored at -20° C. Reactions were normally carried out in a total volume of 30 ul containing the appropriate restriction buffer and 40 ug of RNase A.

The BAL-31 deletion series was screened by restriction of clones with EcoRI and HindIII and electrophoresis of products on a 7% polyacrylamide gel with HinfI digested pBR322 as molecular size markers. The pUC8 clone containing the pV4a.5-45, was digested with HindIII and run on a 0.6% agarose gel. Reclones of pV4a.5-45 were digested with EcoRI and HindIII and run on 6% polyacrylamide gels. The Val_4 5'-flank was isolated from the HindIII site of pUC8 on an 8%

polyacrylamide gel as were the reclones in pEMBL8-. All recombinants carrying the Val_4 pV4a.5-138 or point mutants in pEMBL8- or pTZ19U were digested with EcoRI and HindIII and electrophoresed on 5% polyacrylamide gels to release their inserts.

RESULTS

I. Construction of recombinant plasmids

A. Subcloning of pV4a.5-45

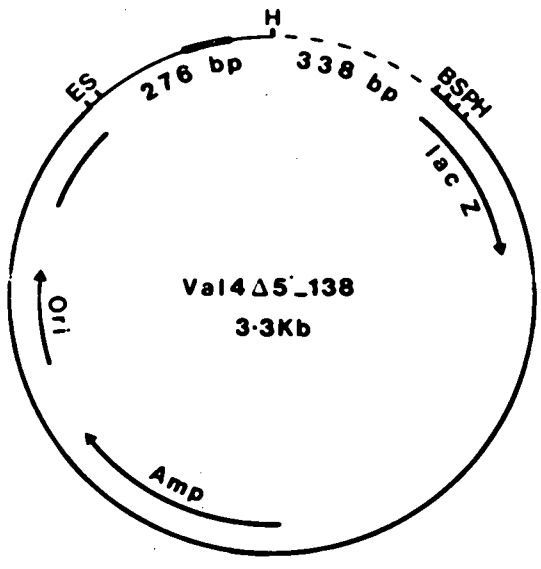
The plasmid used for subcloning was a derivative of plasmid pDt55-0.3. pDt55-0.3 contained a 317 bp *Drosophila* insert coding for $tRNA^{Val}_4$ cloned into the HindIII site of pBR322 in both orientations (Rajput et al., 1982). This plasmid was a derivative of pDt55, which contained two identical $tRNA^{Val}_4$ genes (from 70 BC on the left arm of chromosome 3) 525 bp apart and in opposite polarity (Dunn et al., 1979: Addison et al., 1982). A number of plasmids with deletions in the 5'-flank were created in the 692 bp EcoRI/BamHI fragment of pDt55-0.3 and cloned into the SmaI site of pUC8 (Vieira and Messing, 1982). The 3'-flank of the Val_4 gene was protected from the exonuclease activity of BAL-31 by the 346 bp HindIII/BamHI fragment of pBR322 from pDt55-0.3. The deletion series gave rise to a number of mutants including plasmids pV4a.5-45 and pV4a.5-138 (Figure 1) used in this study (Sajjadi, 1985).

During the analysis of pV4a.5-45, this clone was found to contain two DNA fragments. The plasmid containing the pV4a.5-45 double insert was digested with HindIII and following purification of vector and desired insert from the sequences present at the 3' end of the gene, the plasmid was recircularized and used to transform *E. coli* JM83. Plasmid

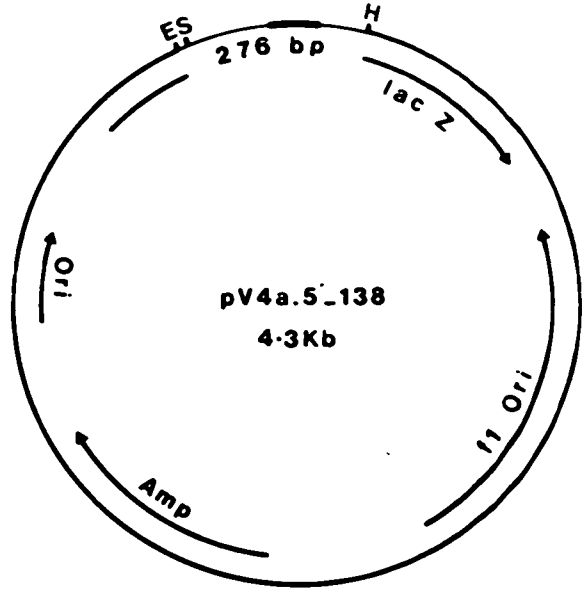
Figure 1 Restriction maps of $Val_4\triangle 5'$-138 and pV4a.5-138

A- The figure shows a BAL-31 deletion derivative of pDt55-0.3. The EcoRI/BamHI fragment containing the gene encoding $tRNA^{Val}_4$ (thick band; 73 bp) 138 bp of 5'-flanking sequence and 65 bp of 3'-flanking sequence was subcloned in the SmaI site of pUC8 along with 338 bp of pBR322.

B- The 276 bp fragment from A was subcloned into the EcoRI/HindIII site of pEMBL8- to generate pV4a.5-138. This construct was used for site-specific mutagenesis in this study.


H
ES
276 bp
338 bp
BSPH
lac Z
Ori
Val4Δ5'_138
3·3Kb
Amp

A


H
ES
276 bp
lac Z
Ori
pV4a.5'_138
4·3Kb
f1 Ori
Amp

B

DNA was isolated and screened by digestion with EcoRI and HindIII. All nine colonies picked contained a single 185 bp insert in plasmid pUC8. Double-stranded DNA from one colony was prepared and sequenced. The sequence demonstrated the presence of a single Val_4 deletion pV4a.5-45. Plasmid DNA was prepared and transcribed and was found to have a Vmax 36% higher than pV4a.5-179 (Sajjadi et al., 1987).

For site-specific mutagenesis of the sequence TCGCT in pV4a.5-45, the 185 bp fragment was isolated by digestion with EcoRI and HindIII and subcloned into EcoRI/HindIII-cut pEMBL8- which can be used to generate single-stranded plasmid DNA (Dente et al., 1983). Ten transformants of pV4a.5-45 in pEMBL8- were picked randomly from plates, each containing ~200 transformants and inoculated into 2 mls of 2YT/amp. Mini-prep plasmid DNA, prepared from these cultures as described in Materials and Methods, was digested with EcoRI and HindIII and analyzed on a 6% polyacrylamide gel by electrophoresis. All clones contained the correct insert. Single-stranded plasmid DNA was prepared from one clone and the insert was sequenced in its entirety using the reverse primer (Pharmacia) to confirm the clone as pV4a.5-45 in pEMBL8-.

B. Subcloning of pV4a.5-138

The pV4a.5-138 clone (276 bp) containing a 338 bp pBR322 fragment in the SmaI site of pUC8 (Sajjadi, 1985) (Figure 1) was digested with EcoRI and HindIII and the 276 bp fragment containing the tRNA gene was isolated after

separation on an 8% polyacrylamide gel. The EcoRI/HindIII fragment was cloned into pEMBL8- as outlined above. Ten transformants were screened by digestion of their plasmid DNA with EcoRI and HindIII and electrophoresis on an 8% polyacrylamide gel. Eight of the clones carried the 276 bp fragment, while two clones contained both the 276 bp and 338 bp fragments. Two of the clones with the 276 bp fragment were sequenced in their entirety to show that they contained pV4a.5-138 in pEMBL8-. In addition, the combination of pUC8 vector and 5'-flanking sequences had recreated the SmaI site in these constructs (Figure 1).

The ligation of the 276 bp EcoRI/HindIII fragment into the EcoRI/HindIII site of the single-stranded plasmid pTZ19U (USB) was carried out as described for the pEMBL8- vector. Four clones were sequenced completely, except that the forward primer was used in sequencing. All clones were found to contain the pV4a.5-138 insert.

C. Subcloning of the pV4a.-179 5'-flank

In a previous study a series of 3' deletions gave rise to clones in the vector pUC8 which contained the intact Val_4 gene and 179 bp of 5'-flanking sequence, but lacked the 3'-flanking region (data not presented). One 3' deletion was further extended to position +2 by D. Horvath (personal communication). The +2 deletion was created in pUC8 with HindIII linkers. However, the construct containing the 5'-flank in pUC8 was found to contain extraneous DNA sequences which could not be identified (data not shown). In order to

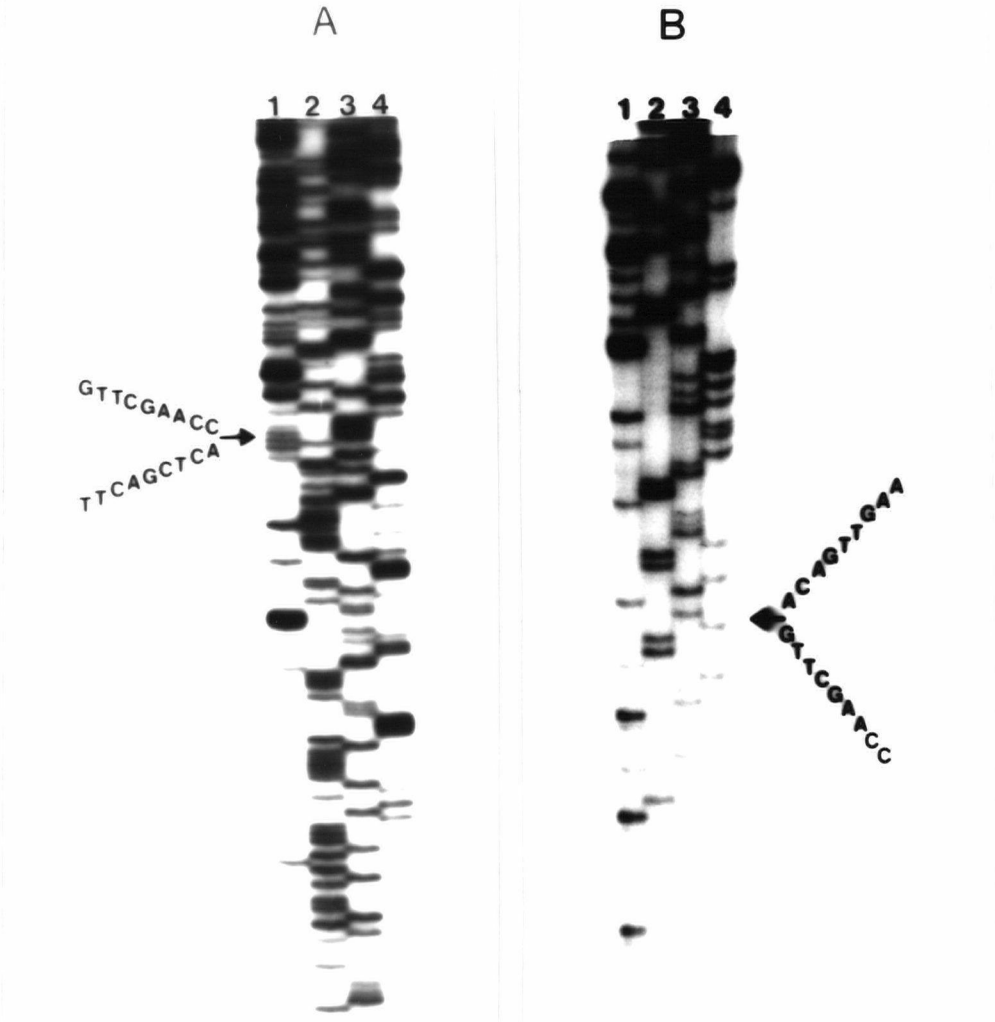
isolate the 5'-flank of the $tRNA^{Val}_4$ gene, the +2 deletion was digested with HindIII and the insert isolated and purified. The 5'-flank fragment was then subcloned into the HindIII site of pEMBL8-. Isolated plasmid DNA from twelve white colonies were screened by digestion with HindIII and ten were found to contain the 5'-flank alone. Single-stranded DNA was isolated from two positive clones and used for sequencing the entire insert with the reverse primer. The sequence confirmed the presence of the isolated 5'-flank of $tRNA^{Val}_4$ gene (Figure 2).

D. Subcloning of the $tRNA^{Ser}_7$ gene and 5'-flanking deletion derivatives

A PvuII/ClaI fragment containing the $tRNA^{Ser}_7$ gene (81 bp), ~420 bp of 5'-flank and 200 bp of 3'-flanking sequences had been cloned into the SmaI site of pEMBL8- to generate pS5#1 (D. St. Louis, personal communication). To remove some of the 5'-flanking sequences, the HhaI site at position -125 relative to the mature coding sequence could be used to delete ~300 bp of 5'-flanking sequence. However, due to the presence of a large number of HhaI sites in pEMBL8, a fragment containing the Ser_7 gene was first subcloned indirectly as follows. The EcoRI/HindIII fragment (~750 bp) containing the entire Drosophila DNA in pS5#1 was isolated from the vector and purified. Next, the isolated fragment was digested with HhaI (a second HhaI site is at

Figure 2 The DNA sequence of isolated 5'-flanking region of pV4a.5-179

The DNA sequence was determined as described in Materials and Methods. Lanes 1-4 represent the sequencing reactions with the reverse primer specific for C, T, A and G respectively. The junction between the vector and 5'-flank is marked by an arrow and the DNA sequence is shown on the side of the autoradiogram.
A- Sequence of the 5' junction of Val_4 5'-flank (position -179, non-coding strand).
B- Sequence of the 3' junction of Val_4 5'-flank (position +2, non-coding strand).

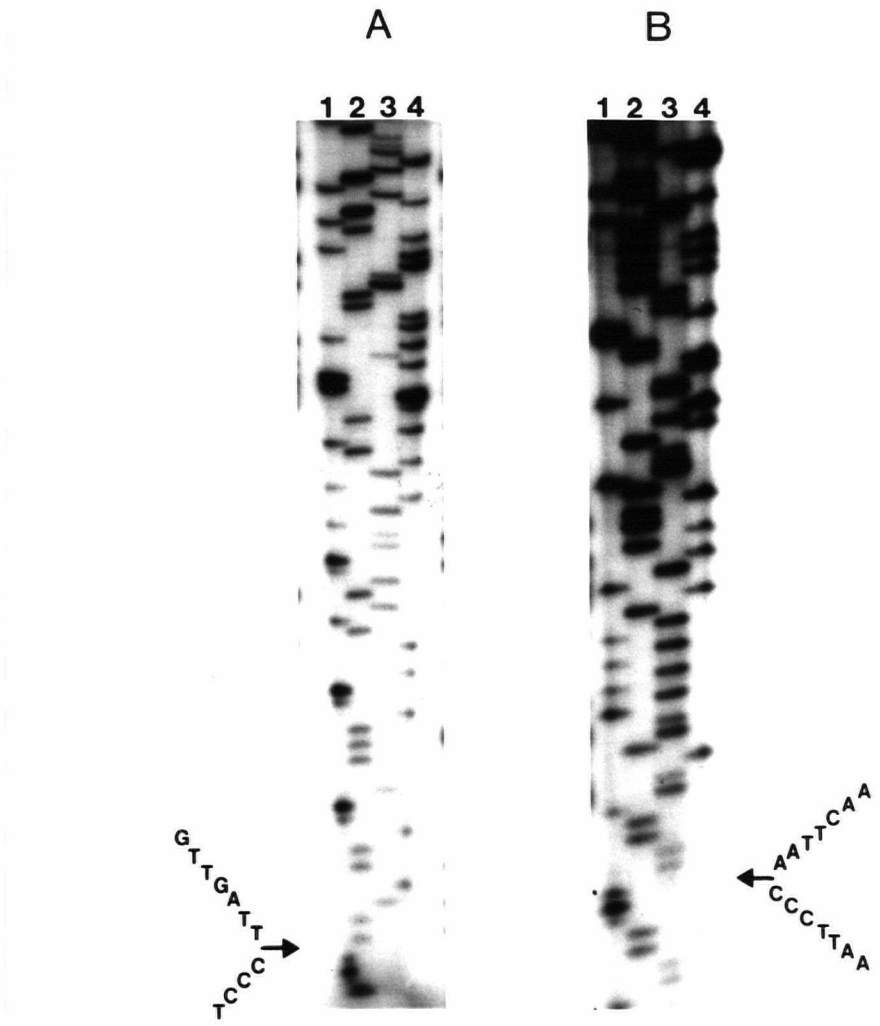

A
B
1 2 3 4
1 2 3 4
GTTCGAACC
TTCAGCTCA
ACAGTTGAA
TGTTCGAACC

position +272) and the fragments were separated on a 5% polyacrylamide gel. The digest resulted in the isolation of a 391 bp HhaI fragment containing the gene, 125 bp of 5'-flanking sequence and 185 bp of 3'-flanking sequence, a ~295 bp EcoRI/HhaI fragment and a ~65 bp HhaI/HindIII fragment. The 391 bp HhaI fragment was recovered from the gel and following a limited BAL-31 exonuclease treatment and separation on a polyacrylamide gel, the deleted fragments were cloned into the SmaI site of pEMBL8. Plasmid DNA was prepared from 24 white colonies and screened by digestion with EcoRI and HindIII and electrophoresed with HinfI-cut pBR322 size markers. Inserts contained in these clones did not appear to have been deleted to a great extent. Five colonies were sequenced in their entirety and none was deleted more than 6 bp in the 5'-flank. Interestingly, the 3'-flank of these constructs was not deleted beyond position +266, indicating that only the sequences from the multiple cloning site of pEMBL had been removed. Therefore, only two clones were obtained from this deletion series: pS7a.5-123F and pS7a.5-119R (Figure 3) (F and R refer to the orientation of the insert: in the R position, the 5' end of the coding strand of the gene is closest to the reverse sequencing priming site).

To extend the 5'-flanking deletion series closer to the $tRNA^{Ser}_7$ gene, the Drosophila insert contained in pS7a.5-119 was isolated after digestion of plasmid DNA with EcoRI and HindIII and was subjected to further exonuclease action

Figure 3 The DNA sequences of pS7a.5-119 (A) and pS7a.5-31 (B)

The DNA sequences were determined as described in Materials and Methods. Lanes 1-4 represent the sequencing reactions with the reverse primer specific for C, T, A and G respectively. The junction between the vector and 5'-flank is marked by an arrow and the DNA sequence is shown on the side of the autoradiograms.


A
B
1 2 3 4
1 2 3 4
GTTGATT
TCCC
AATTCAA
CCCTTAA

by BAL-31. This scheme would prevent the migration of deleted fragments from the vector into the areas of gel containing deleted, liberated insert molecules had a different approach involving Bal-31 deletion of linear plasmids been adopted. The smearing was a significant problem since the deleted vector molecules contaminating the deleted insert fragments were cloned in preference to the insert, thus reducing the cloning efficiency of the desired fragments.

Deleted fragments were cloned into the SmaI site of pEMBL8- and used to transform E. coli NM522. Forty white colonies were screened as described in Materials and Methods (Figure 4). Clones which appeared to contain deleted insert were sequenced in their entirety. The following deletion end-points were obtained: pS7a.5-98R, pS7a.5-31F and R (Figure 3), pS7a.5-26F, pS7a.5-24R and pS7a.5-18F (Figure 5). All clones were found to retain 184 bp of 3'-flanking sequence.

II. Site-specific mutagenesis

A. pV4a.5-45

The pV4a.5-45 clone in pEMBL8- was used for oligonucleotide-directed mutagenesis (Zoller and Smith, 1984; Kunkel, 1985) using a 17mer oligonucleotide containing a single mismatch with the template. Three factors were found to influence mutagenesis efficiency. First, the standard 16 hr primer extension was found to result in few or no transformant colonies. Extension reaction times were

Figure 4 Screening of deletion mutants

A 7% polyacrylamide gel containing 12 cloned deletion derivatives of pS7a.5-119 is shown. Following mini-prep isolation of plasmid DNAs and digestion with EcoRI and HindIII, products were electrophoresed along with 2 ug of HinfI cut pBR322 (H).

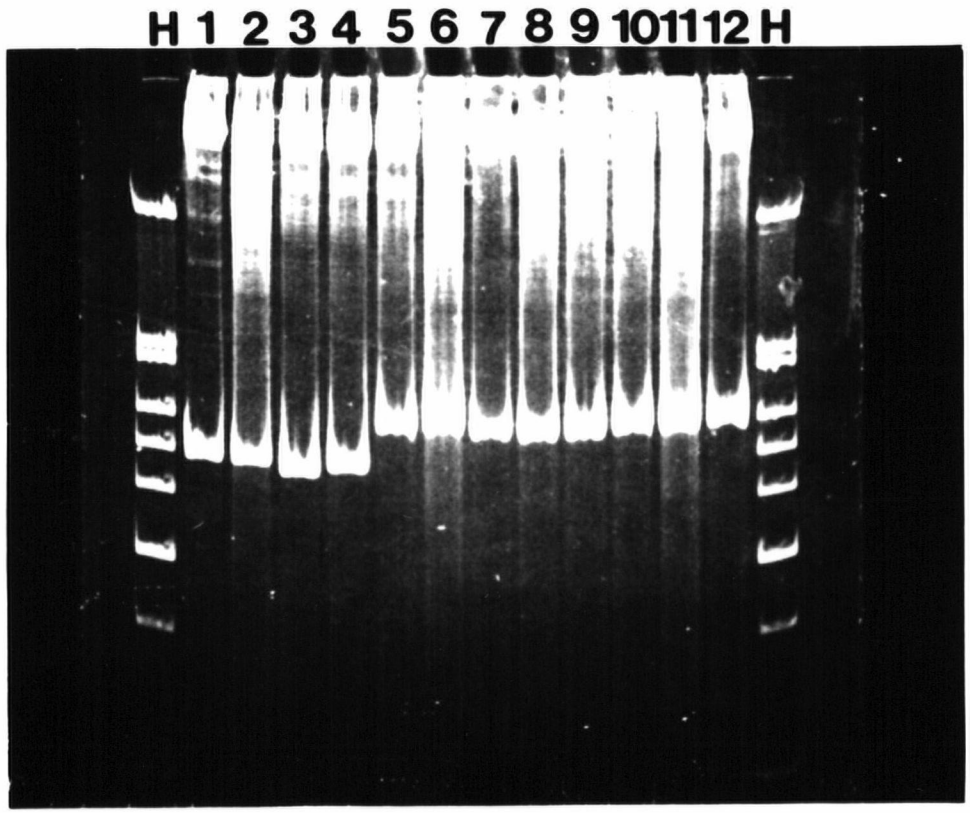
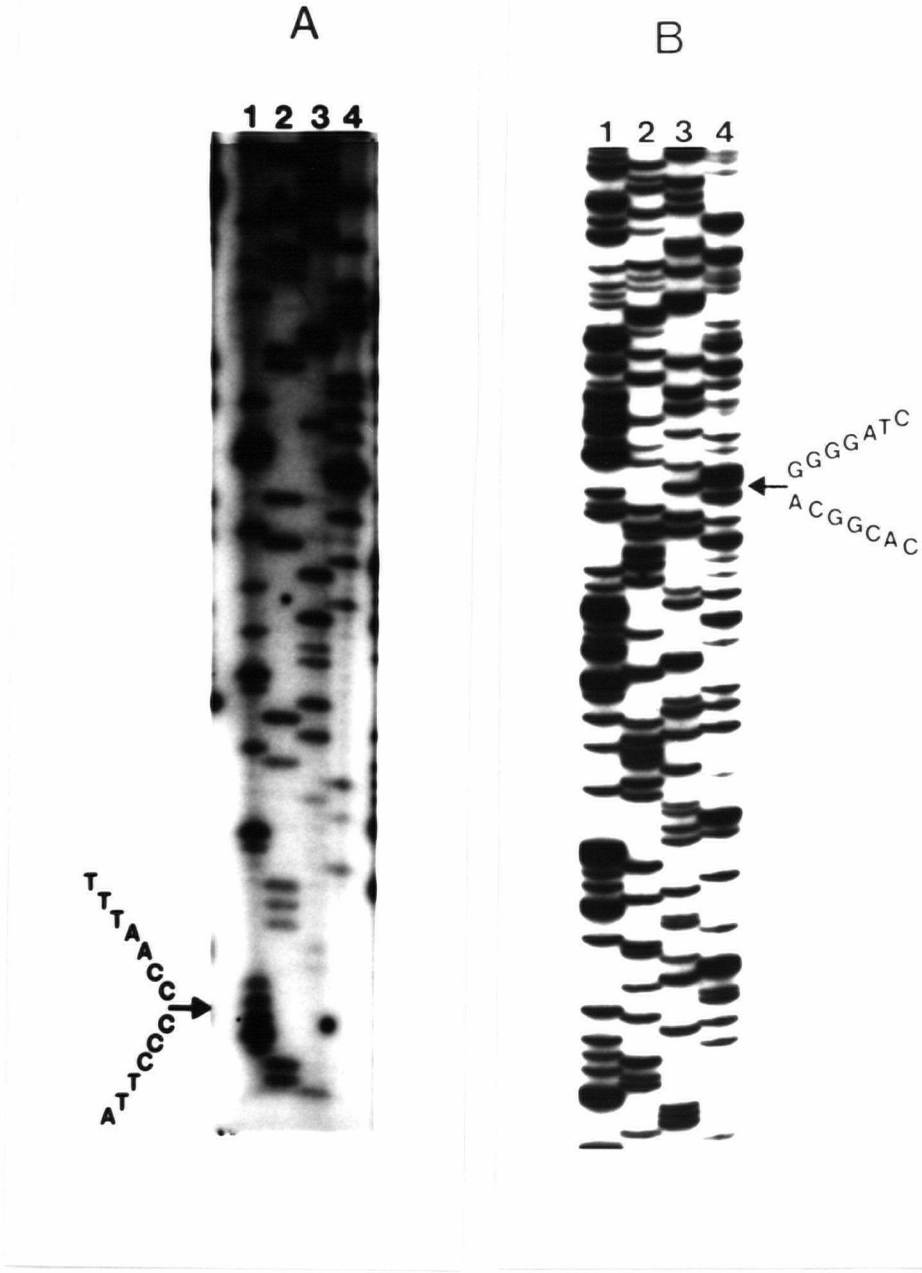

H 1 2 3 4 5 6 7 8 9 10 11 12 H

Figure 5 The DNA sequences of pS7a.5-24 (A) and pS7a.5-18 (B)

The DNA sequences were determined as described in Materials and Methods. Lanes 1-4 represent the sequencing reactions with the reverse primer specific for C, T, A and G respectively. The junction between the vector and 5'-flank is marked by an arrow and the DNA sequence is shown on the side of the autoradiograms. The non-coding strand of deletion end-point -18 was sequenced.


A
1 2 3 4
TTTAACCCCCCATTCCC
B
1 2 3 4
GGGGATC
ACGGCAC

shortened as described in Materials and Methods. Second, the commercial source of Klenow polymerase and DNA ligase were found to influence the recovery of transformant colonies. Another group had found their source of DNA ligase responsible for decreased frequencies of transformants (Naumovski and Feinberg, 1986). Several commercial sources of Klenow and DNA ligase were tested to find the source resulting in the best transformation frequencies. Finally, a 12 bp region in the f1 origin of replication of pEMBL8- was found to hybridize to the mutagenic oligonucleotide. This became apparent by the appearance of a secondary sequence when the mutagenic oligonucleotide was used as a primer for sequence analysis on the pV4a.5-45 template. In addition, a test of priming to the secondary hybridization site was carried out by primer extension of both single-stranded pV4a.5-45 and M13K07 helper phage (1 hr at 25° C) and electrophoresis of reaction products on a 0.7% agarose gel. The extension of hybridized primer would convert the template to double-stranded form which would migrate to a different position on the gel from single-stranded template. It was found that both the pV4a.5-45 clone and the helper phage single-stranded DNA were primed and retarded on the gel (data not shown). Despite the apparent primer extension from the secondary hybridization site, mutagenesis of the pV4a.5-45 clone resulted in the appearance of very few colonies per

plate. Eleven colonies were sequenced with the reverse primer to obtain two mutated clones.

Dideoxy sequence analysis of the mutant clone showed a T to G transversion as a compression in the sequence GGGCGCT which had resulted from the contribution of a G-C rich sequence at the cloning site of pV4a.5-45. The sequence compression was resolved by Maxam and Gilbert chemical sequencing as described in Materials and Methods (Figure 6).

B. pV4a.5-138

The pV4a.5-138 clone in pEMBL8- was used for oligonucleotide directed mutagenesis of $^{-38}$TCGCT^{-34} in its 5'-flank using an 18mer mixed oligonucleotide. As in section II.A. very few transformant colonies were obtained and of the 15 clones sequenced, two were found to have single point mutations. Only a T to G transversion at position -38 was recovered. Neither of these mutants showed sequence compression in the -38 region when compared to pV4a.5-45 (Figures 6 and 7).

The extension of reverse primer and mutagenic oligonucleotides in pEMBL8- occurred in close proximity to the f1 origin of replication (the site of secondary hybridization identified in section A) which impeded second strand synthesis. Because single-stranded DNA is very inefficient as a substrate for transformation, it was reasoned that primer extension away from the secondary hybridization site would result in plasmid molecules with a more extensive double-stranded region and hence a

Figure 6 The DNA sequence of pV4a.5-45,-38G

The DNA sequences were determined as described in Materials and Methods.
A- Lanes 1-4 represent the dideoxy sequencing reactions with the reverse primer specific for C, T, A and G respectively. The sequence compression is marked by an arrow and the DNA sequence is shown on the side of the autoradiogram.
B- Lanes 1-4 represent the Maxam and Gilbert sequencing reactions specific for C, C+T, A+G and G respectively. The DNA sequence is shown on the side of the autoradiogram. The sequence reads in the 3' to 5' direction.

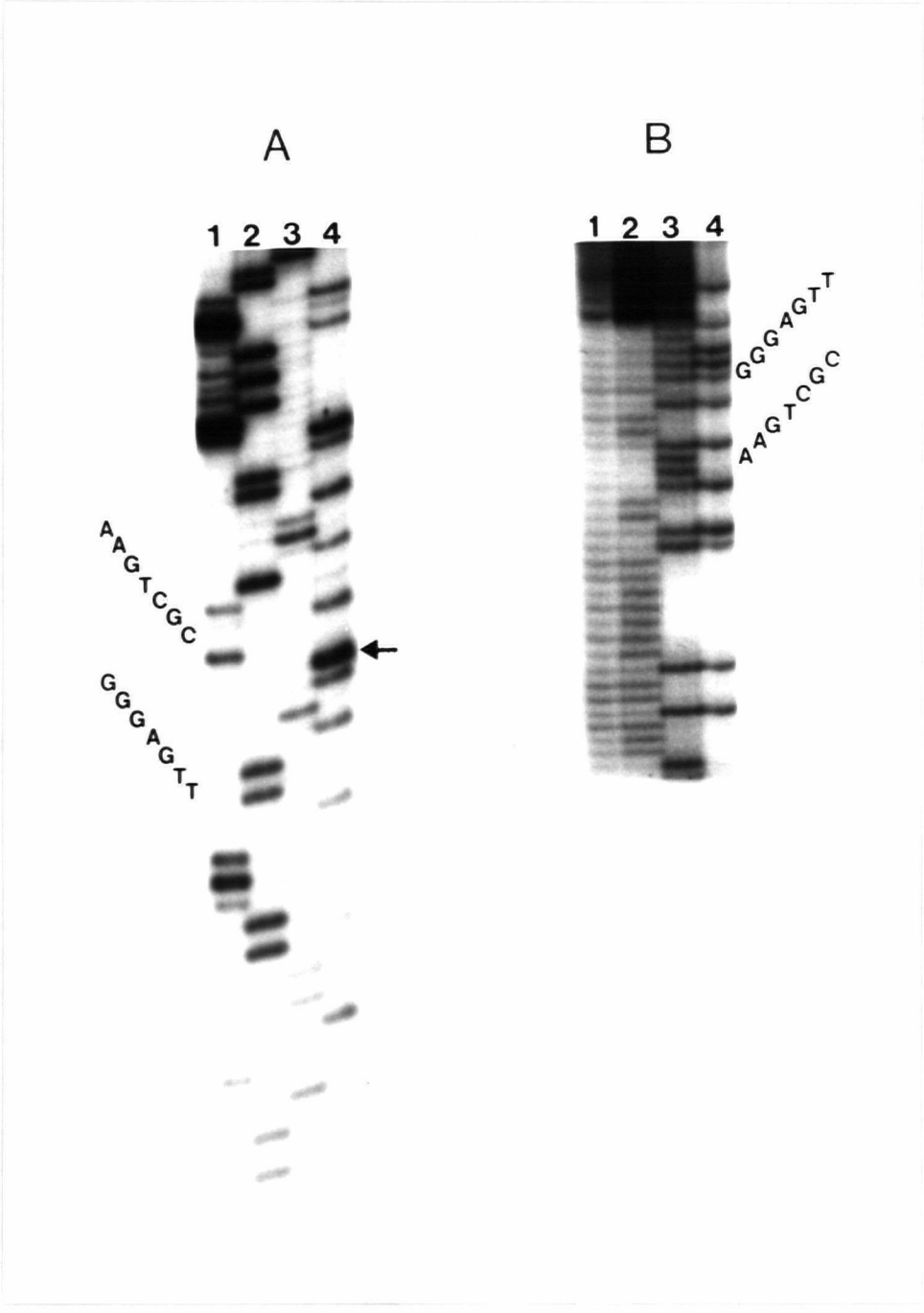
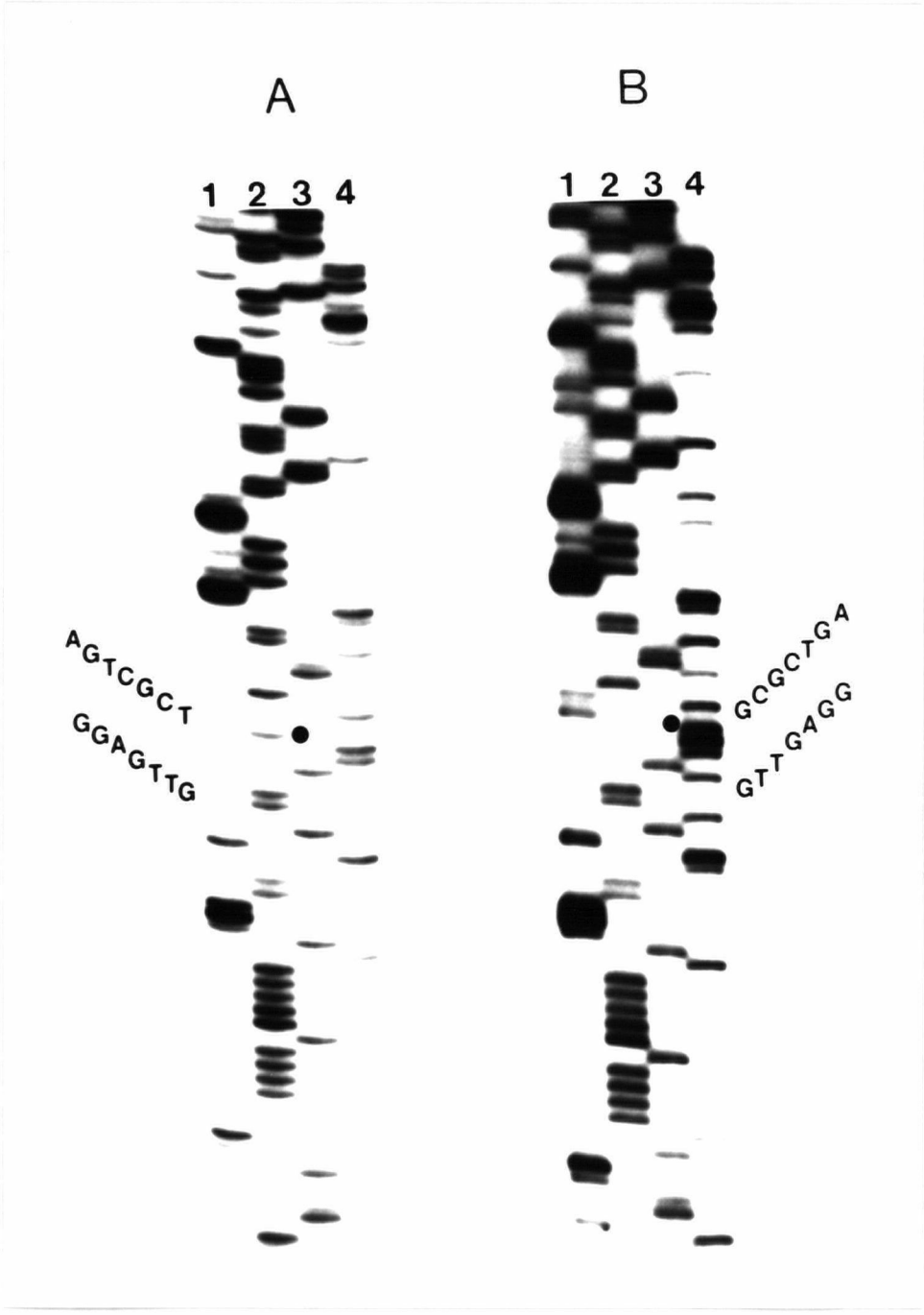

A
1 2 3 4
AAGTCGC
GGGAGTT
B
1 2 3 4
GGGAGTT
AAGTCGC

Figure 7 The DNA sequences of pV4a.5-138 (A) and pV4a.5-138,-38G (B)

The DNA sequences were determined as described in Materials and Methods. Lanes 1-4 represent the sequencing reactions with the reverse primer specific for C, T, A and G respectively. The sequences of wild type and mutant TNNCTs are shown on the side of the autoradiograms. The band corresponding to position -38 are indicated by a dot on the autoradiogram.


A
B
1 2 3 4
1 2 3 4
AGTCGCT
GGAGTTG
GCGCTGA
GTTGAGG

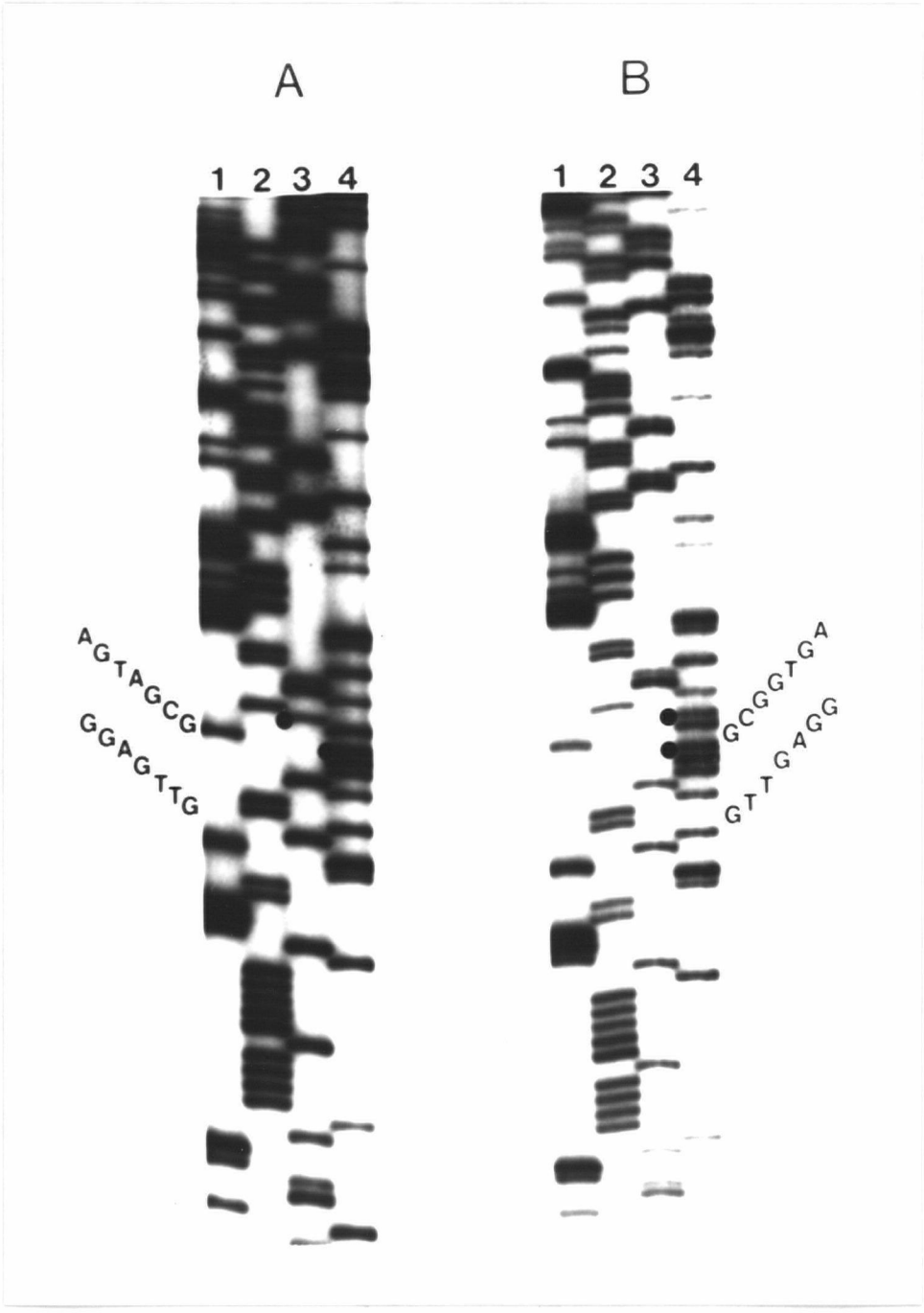
greater transformation efficiency. Since the site of secondary hybridization showed homology with sequences between positions -42 and -27 in the 5'-flank of $tRNA^{Val}_4$ gene, it was not possible to shift effectively the priming site of the mutagenic oligonucleotide either 3' or 5' to $^{-38}$TCGCT^{-34} in order to minimize homology with the second site at the f1 origin of replication. The EcoRI/HindIII insert contained in pV4a.5-138 in pEMBL8- was therefore subcloned into the EcoRI/HindIII site of pTZ19U. Due to the orientation of the insert in pTZ19U, the mutagenic primer could be extended in the direction away from the f1 origin of replication.

Site-specific mutagenesis was carried out on pV4a.5-138 in pTZ19U with the mixed oligonucleotide. Dideoxy sequence analysis of eight clones with the forward primer revealed two single point mutants, both having a T to G transversion at position -38. Thus, a mutagenic bias appeared to exist with the mixed oligonucleotide and other mixed oligonucleotides were not used in later mutagenesis experiments. The mutated Drosophila DNA from pTZ19U was not subcloned in pEMBL8- prior to transcription analysis since the -38G mutant had already been created in pEMBL8-.

A 33% mutagenesis efficiency was achieved for recovery of the double-mutant $^{-38}$GCGAT^{-34} (2 of 6 transformants sequenced) (Figure 8). A second 19mer mutagenic oligonucleotide was used to produce the double-mutant $^{-38}$GCGGT^{-34} using the $^{-38}$GCGCT^{-34} mutant as template (Figure

Figure 8 The DNA sequences of pV4a.5-138,-38G,-35A (A) and pV4a.5-138,-38G,-35G (B)

The DNA sequences were determined as described in Materials and Methods. Lanes 1-4 represent the sequencing reactions with the reverse primer specific for C, T, A and G respectively. The sequences of mutant TNNCTs are shown on the side of the autoradiograms. The bands corresponding to positions -38 and -35 are indicated by dots on the autoradiograms.


A
B
1 2 3 4
1 2 3 4
AGTAGCG
GGAGTTG
GCGGTGA
GTTGAGG

8). All other point mutants were generated with the wild type template. A 21mer oligonucleotide was used to create $^{-38}$TCGAT^{-34} with a 50% mutagenesis frequency (Figure 9). For the creation of $^{-38}$TAGCT^{-34}, $^{-38}$TCTCT^{-34} and $^{-38}$TCGCA^{-34}, 22mer oligonucleotides were used, each of which resulted in an efficiency of 75% mutants. This more closely reflected the efficiencies expected from this procedure (Kunkel, 1985). All mutants were subcloned into the pEMBL8- vector and resequenced to ensure presence of the expected mutations in the 5'-flank (Figures 9 and 10).

Since the longer length of mutagenic oligonucleotides had resulted in higher mutation frequencies, a 32 nucleotide mutagenic primer was used to create three nucleotide changes in the sequence $^{+29}$TGCCT^{+33} in pV4a.5-138 (Figure 11) and pV4a.5-138,-38G,-35A (Figure 12) contained in pEMBL8-. The oligonucleotide was found not to hybridize to sequences contained in the vector. Single-stranded template DNA of two transformants from pV4a.5-138 and two from pV4a.5-138, -38G,-35A were sequenced with the reverse primer. One half the clones recovered were found to contain the three nucleotide changes desired ($^{+29}$AGCGC^{+33}) between the D and T control regions (Figure 13).

Figure 9 The DNA sequences of pV4a.5-138,-34A (A) and pV4a.5-138,-35A (B)

The DNA sequences were determined as described in Materials and Methods. Lanes 1-4 represent the sequencing reactions with the reverse primer specific for C, T, A and G respectively. The sequences of mutant TNNCTs are shown on the side of the autoradiograms. The bands corresponding to positions -34 and -35 are indicated by dots on the autoradiograms.

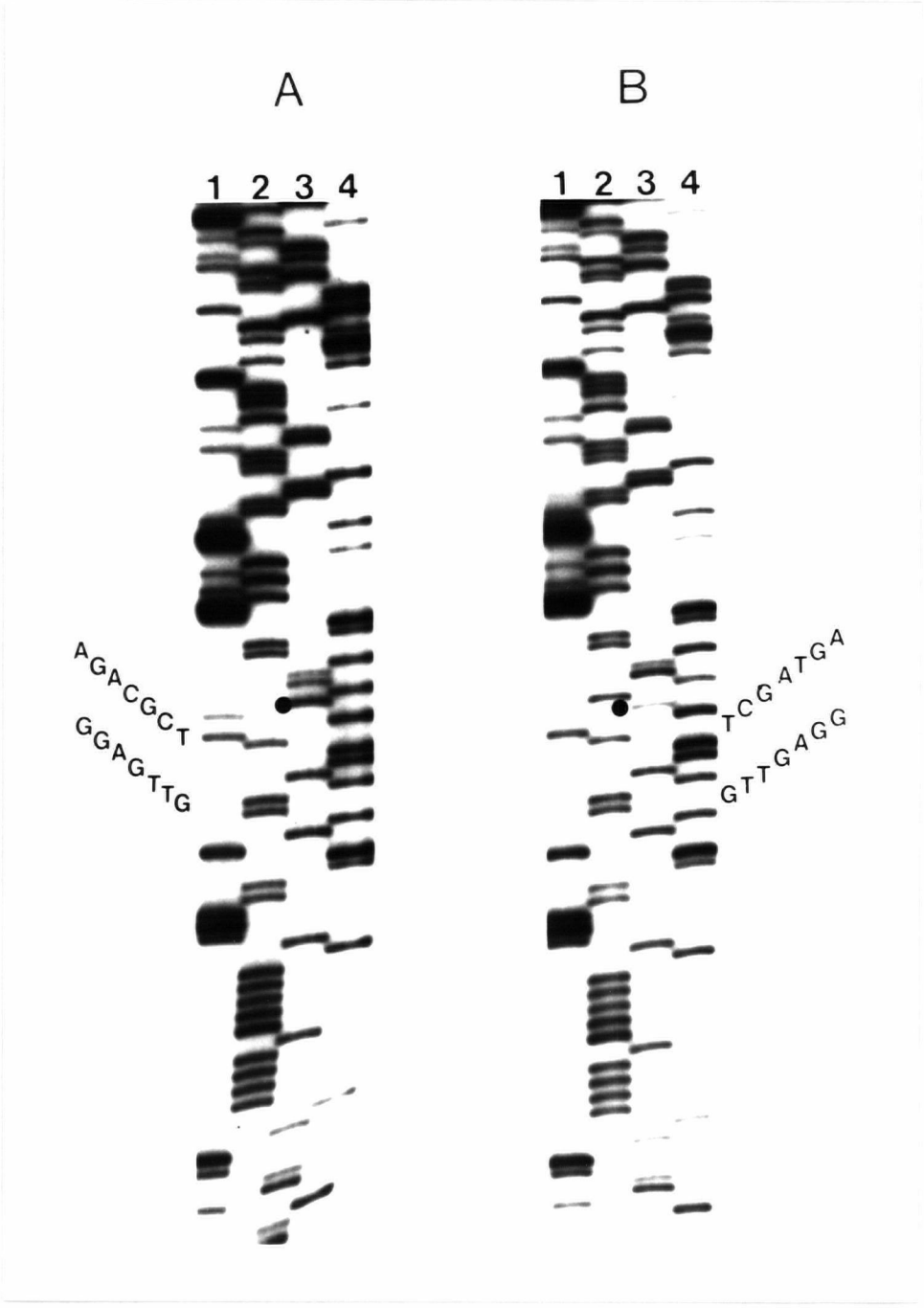

A
B
1 2 3 4
1 2 3 4
AGACGCT
GGAGTTG
TCGATGA
GTTGAGG

Figure 10 The DNA sequences of pV4a.5-138,-36T (A) and pV4a.5-138,-37A (B)

The DNA sequences were determined as described in Materials and Methods. Lanes 1-4 represent the sequencing reactions with the reverse primer specific for C, T, A and G respectively. The sequences of mutant TNNCTs are shown on the side of the autoradiograms. The bands corresponding to positions -36 and -37 are indicated by a dot on the autoradiograms.

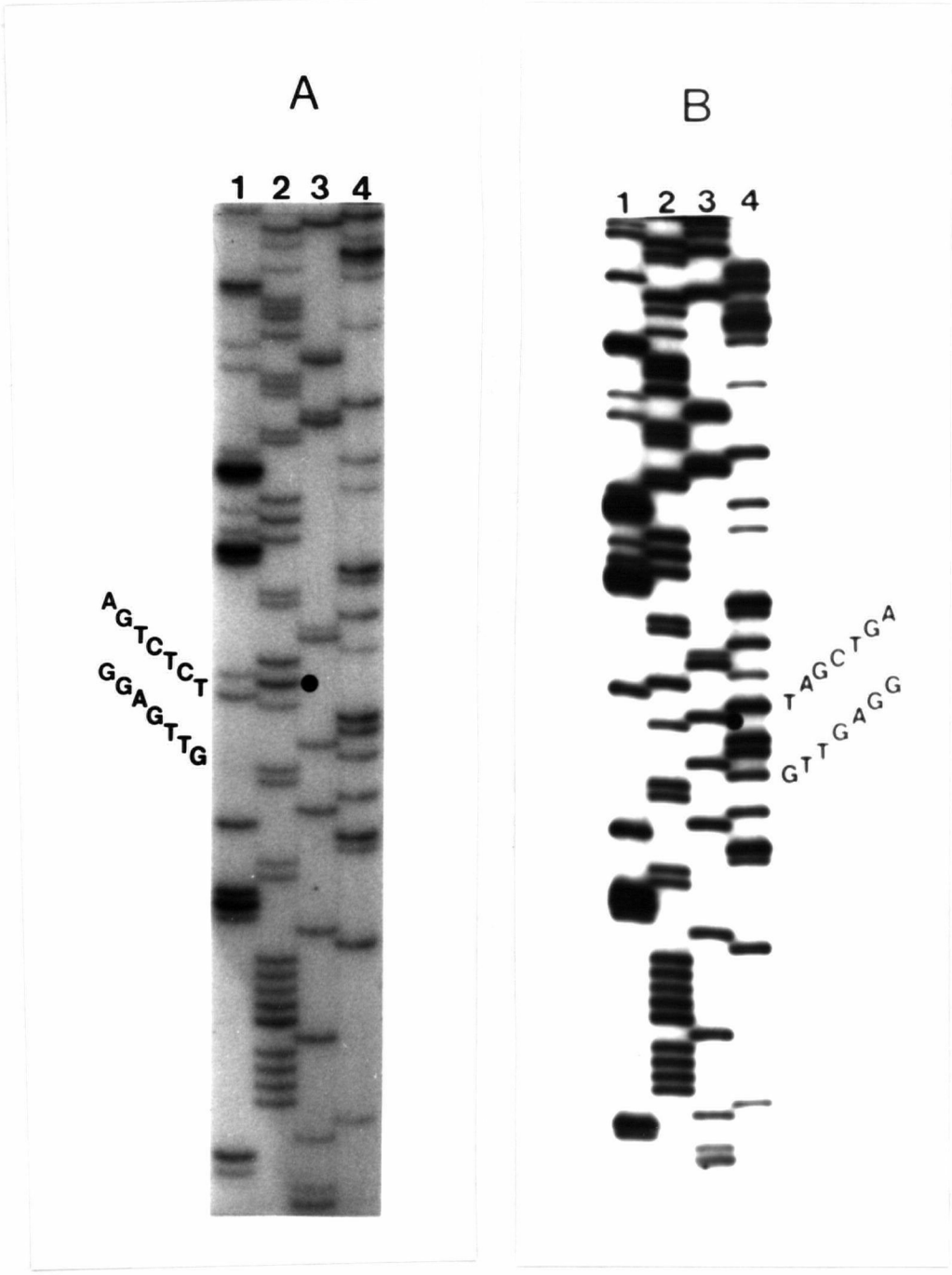

A
1 2 3 4
AGTCTCT
GGAGTTG
B
1 2 3 4
TAGCTGA
GTTGAGG

Figure 11 The DNA sequences of pV4a.5-138 wild type (A) and pV4a.5-138,+29A,+32G,+33C (B)

The DNA sequences were determined as described in Materials and Methods. Lanes 1-4 represent the sequencing reactions with the reverse primer specific for C, T, A and G respectively. The sequences of wild type and mutant internal TNNCTs are shown on the side of the autoradiograms. Arrow heads mark the position of TNNCT in the 5'-flank. The bands corresponding to positions +29 are indicated by a dot on the autoradiograms.

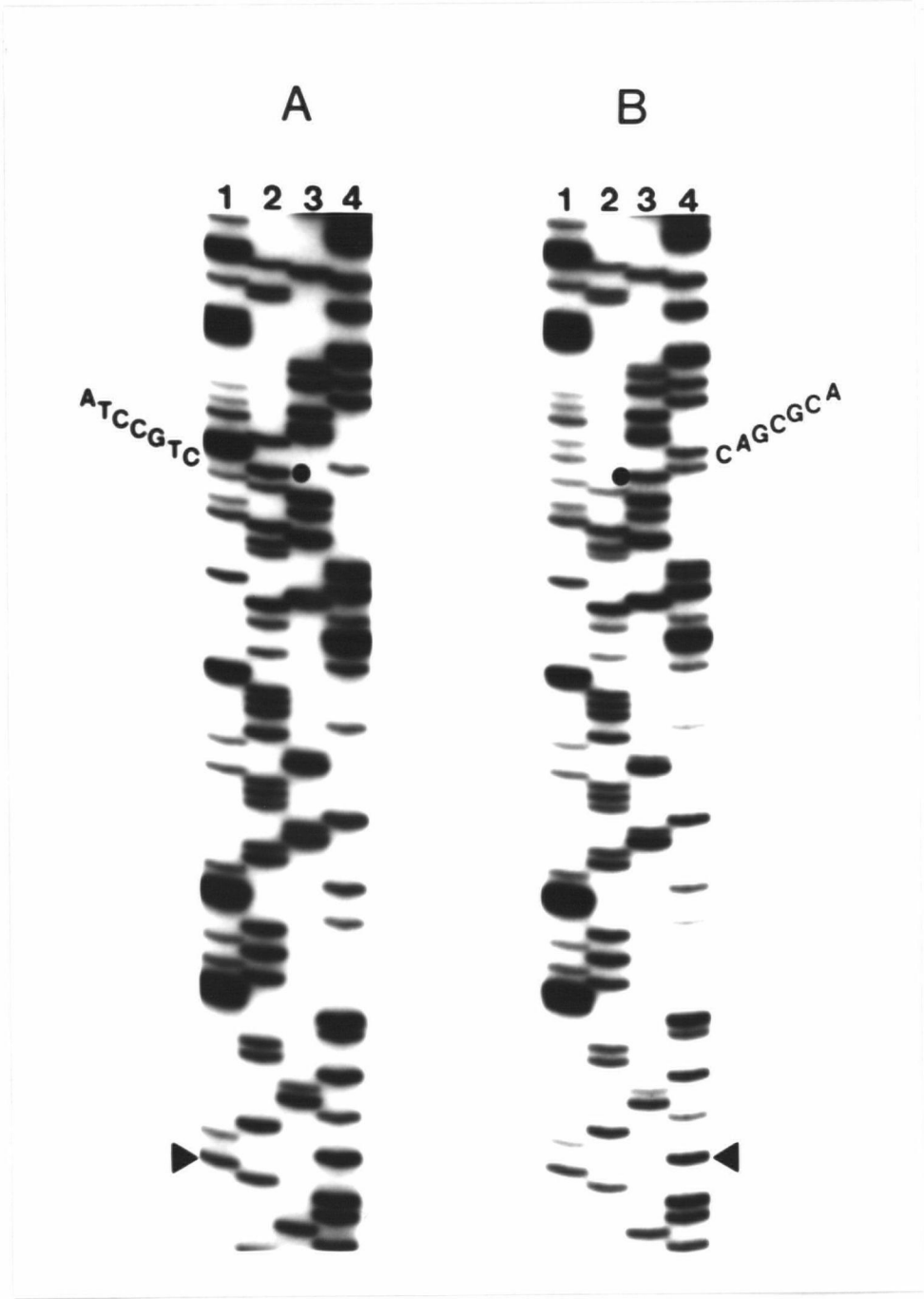

A
B
1 2 3 4
1 2 3 4
ATCCGTC
CAGCGCA

Figure 12 The DNA sequences of pV4a.5-138,-38G,-35A (A) and pV4a.5-138,-38G,-35A,+29A,+32G,+33C (B)

The DNA sequences were determined as described in Materials and Methods. Lanes 1-4 represent the sequencing reactions with the reverse primer specific for C, T, A and G respectively. The sequences of wild type and mutant internal TNNCTs are shown on the side of the autoradiograms. Arrow heads mark the position of -38G,-35A in the 5'-flank. The bands corresponding to position +29 are indicated by a dot on the autoradiograms.

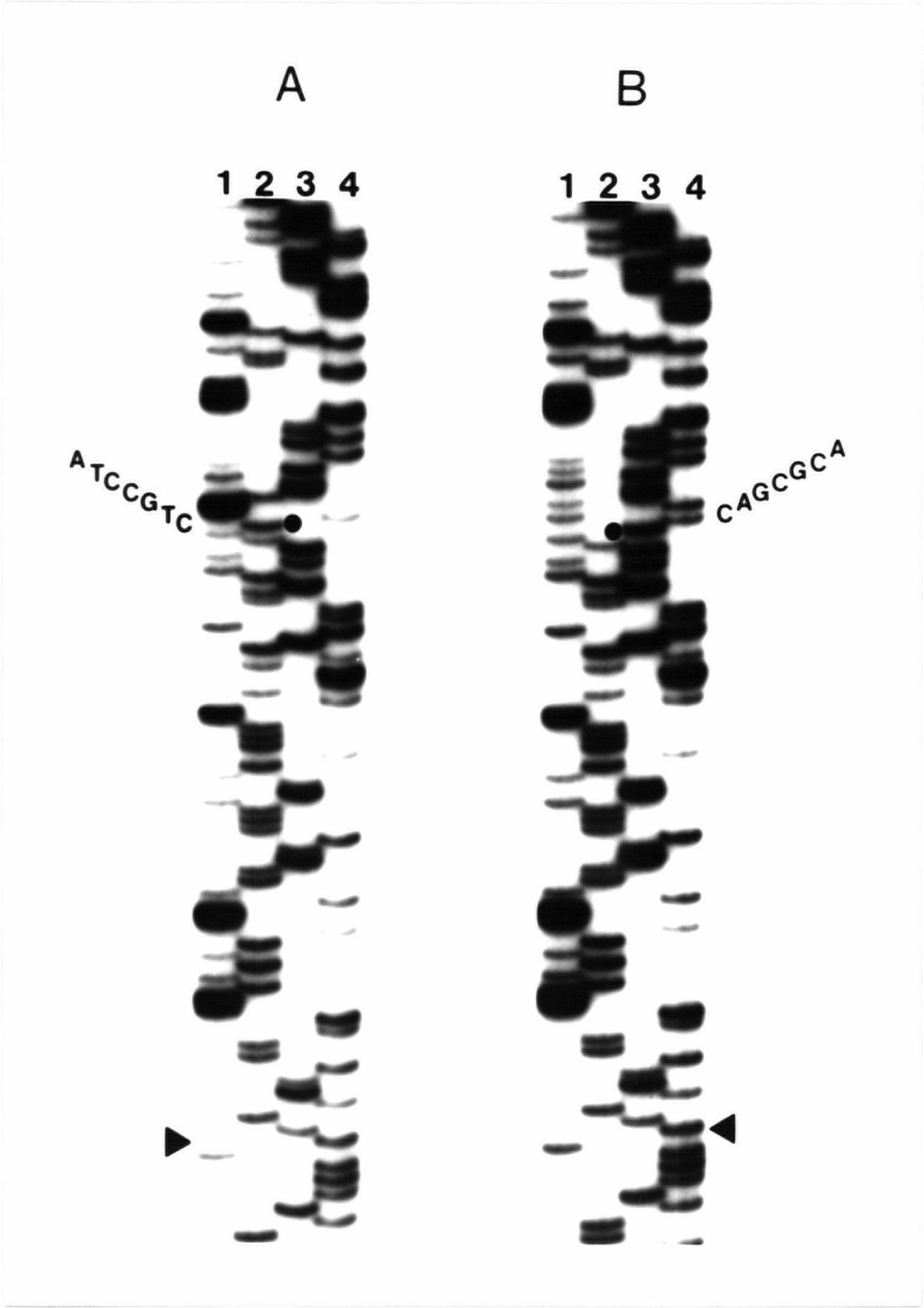

A
B
1 2 3 4
1 2 3 4
ATCCGTC
CAGCGCA

Figure 13 The sequence of pV4a.

Sequence of the non-coding strand of pV4a. is shown to position -140. The 3'-flanking region following poly T termination signal is not shown. The sequence AGTTG is underlined and the TNNCTs are double underlined. The first nucleotide of the mature $tRNA^{Val}_{4}$ gene, the D and T control sequences are in bold letters.
The nucleotide sequence of $tRNA^{Val}_{4}$ arranged as a cloverleaf is shown below the sequence.

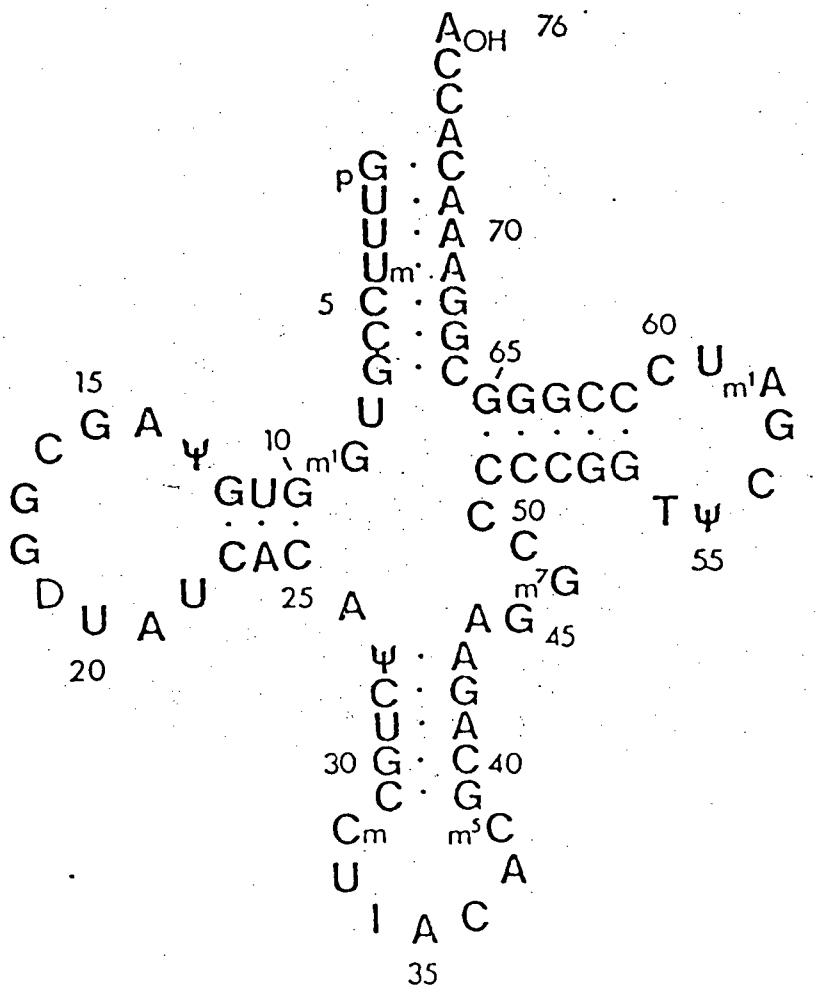
-140 TTGGGTCTCCTTGAAACATTTCCCATAAAAATCACTCAAATAGATACAATA -90

-89 TACGATTTTATTCAAGCAACCAGTTTTATTTTTGACCCTTGGCAGTTGAGG -39

-38 TCGCTGAAGTTGGCCTCTCTGCCGCTTAAGTTTCAACTGTTTCCGTGGTGT +13

+14 AGCGGTTATCACATCTGCCTAACACGCAGAAGGCCCCCGGTTCGATCCCGG +64

+65 GCGGAAACAGGTGATAAACTTTTTTTTTAGTTTTT +119



III. Transcription of mutant TNNCTs and deletion derivatives in a Drosophila (Schneider II) cell-free extract

A. 5'-flanking TNNCT mutants

Point mutants in the 5'-flank of $tRNA^{Val}_4$ genes were transcribed *in vitro* in a homologous Schneider II S-100 cell-free extract as described in Materials and Methods. In all transcriptions pUC8 or pUC13 DNA was added to a total of 1 ug per reaction to counter an inhibitor reported to be present (St. Louis and Spiegelman, 1985; Sajjadi, 1985). Following a 90 min reaction time, the product was separated from the reaction mix on polyacrylamide gels and the amount of transcription product determined by Cerenkov counting the appropriate gel slices. Values corresponding to the velocity of transcription (V= cpm of product per 90 min reaction time= rate of reaction) were corrected to cpm per hour and analyzed by the method of St. Louis and Spiegelman (1985). The method assumes that the kinetics of transcription conform to those of a classic one substrate enzyme reaction and can be analyzed by the Lineweaver-Burke method. The reciprocal of the values for velocity of the reaction and substrate input (S= ug of template DNA) were plotted and the Vmax was derived by linear-regression of 1/V vs 1/S data. To counter day to day variations, transcription of the mutant templates was compared to transcription of pV4a.5-45 or pV4a.5-138 performed in parallel.

The values for Vmax were expressed as percent increase or decrease of the value of Vmax for pV4a.5-45 or pV4a.5-138 transcribed on the same day. Each template containing the mutant TNNCT sequences was transcribed with two different active S-100 cell-free extracts to test the variability in transcription efficiency between different S-100 preparations (Sajjadi, 1985). However, the percent increase or decrease in Vmax, when compared to the wild type gene, for all point mutants was nearly the same in all extracts. The percent increase or decrease in Vmax for each TNNCT mutant was calculated and the average of the two values from the two different extracts was reported (Table 1). An example of the double-reciprocal plot obtained with data from the transcriptions of pV4a.5-138 and pV4a.5-138, -38G,-35A is presented in Figure 14-B. The Vmax could be estimated graphically from the intercepts. However, they were calculated numerically from the intercepts of least squares lines describing the data.

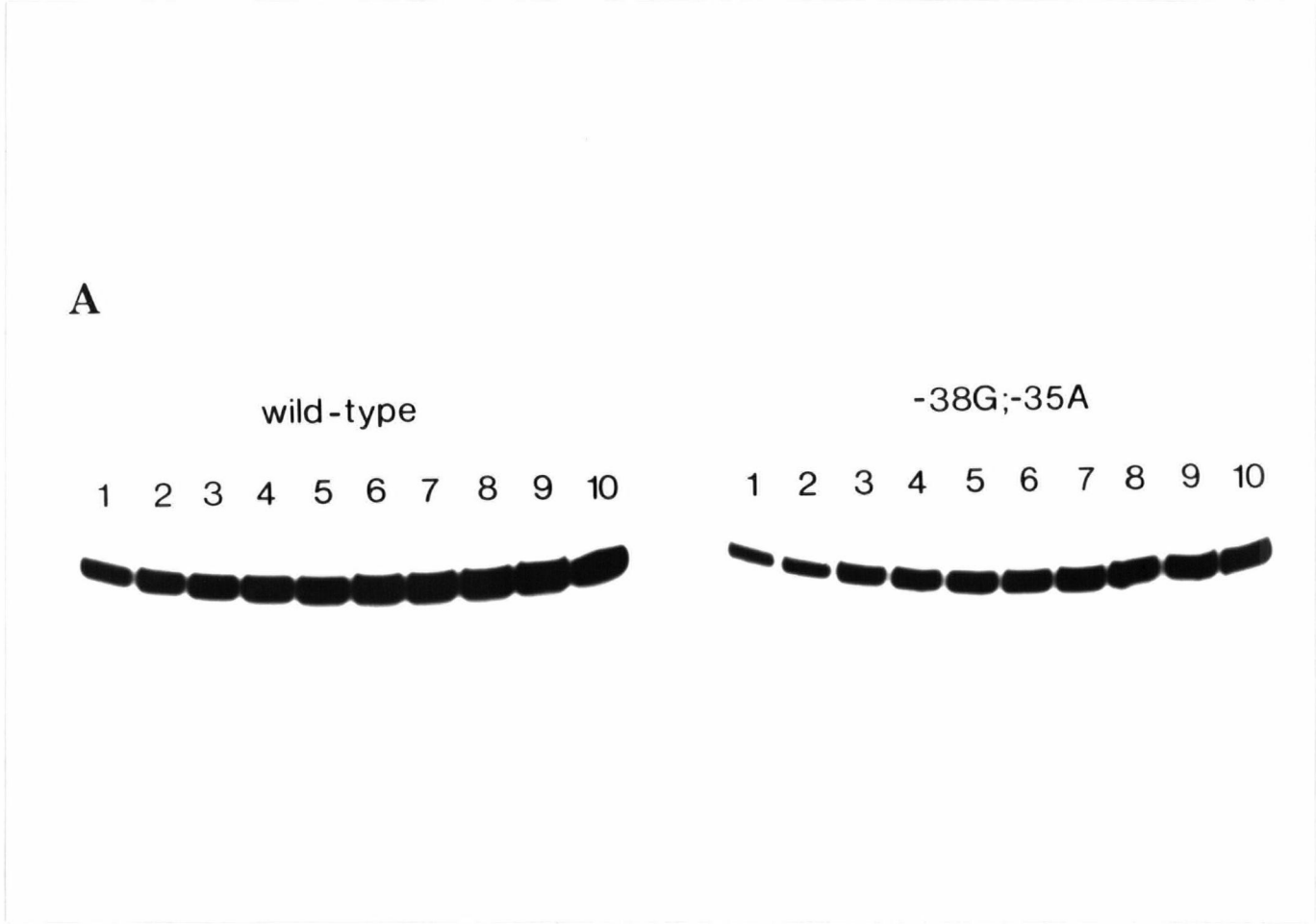
Figure 15 shows 12-point input transcriptions ranging from 3 to 150 ng of DNA for pV4a.5-45,-38G and its control. The Vmax for the transversion was decreased 10%. In contrast to pV4a.5-45,-38G, the -38G mutation in pV4a.5-138 (Figure 16) resulted in a 28.2% decrease in Vmax. Point mutants at the other conserved nucleotides of TNNCT (-34A and -35A) resulted in a slightly greater drop in Vmax than for -38G (Table 1; Figures 17 and 18). Double point mutants displayed the lowest template activities with a decreased

Figure 14A Autoradiogram of products from the transcription of pV4a.5-138 (wild type) and pV4a.5-138,-38G, -35A

Autoradiograms representing the electrophoretic separation of labelled tRNAs from pV4a.5-138 (wild type) and pV4a.5-138,-38G,-35A are shown. Transcriptions were carried out using a total DNA concentration of 1.0 ug per reaction at 23.5° C and transcription products were analyzed on polyacrylamide gels as described in Materials and Methods. Lanes 1-10 show the transcription products of 10, 15, 20, 25, 33, 40, 50, 66.7, 84 and 100 ng of template DNA.

B Double reciprocal plot of data from transcription of pV4a.5-138 (wild type) (▲) and pV4a.5-138,-38G,-35A (■)

Gel slices corresponding to transcription products were excised and the amount of product was determined by Cerenkov counting. The data were plotted as 1/V (V= cpm of transcript/hr of reaction) versus 1/S (S= mass of template in ug). The lines were derived by the method of least squares with coefficients of 0.998 for pV4a.5-138 wild type (▲) and 0.999 for pV4a.5-138,-38G,-35A (■).


A
wild-type
1 2 3 4 5 6 7 8 9 10
-38G;-35A
1 2 3 4 5 6 7 8 9 10

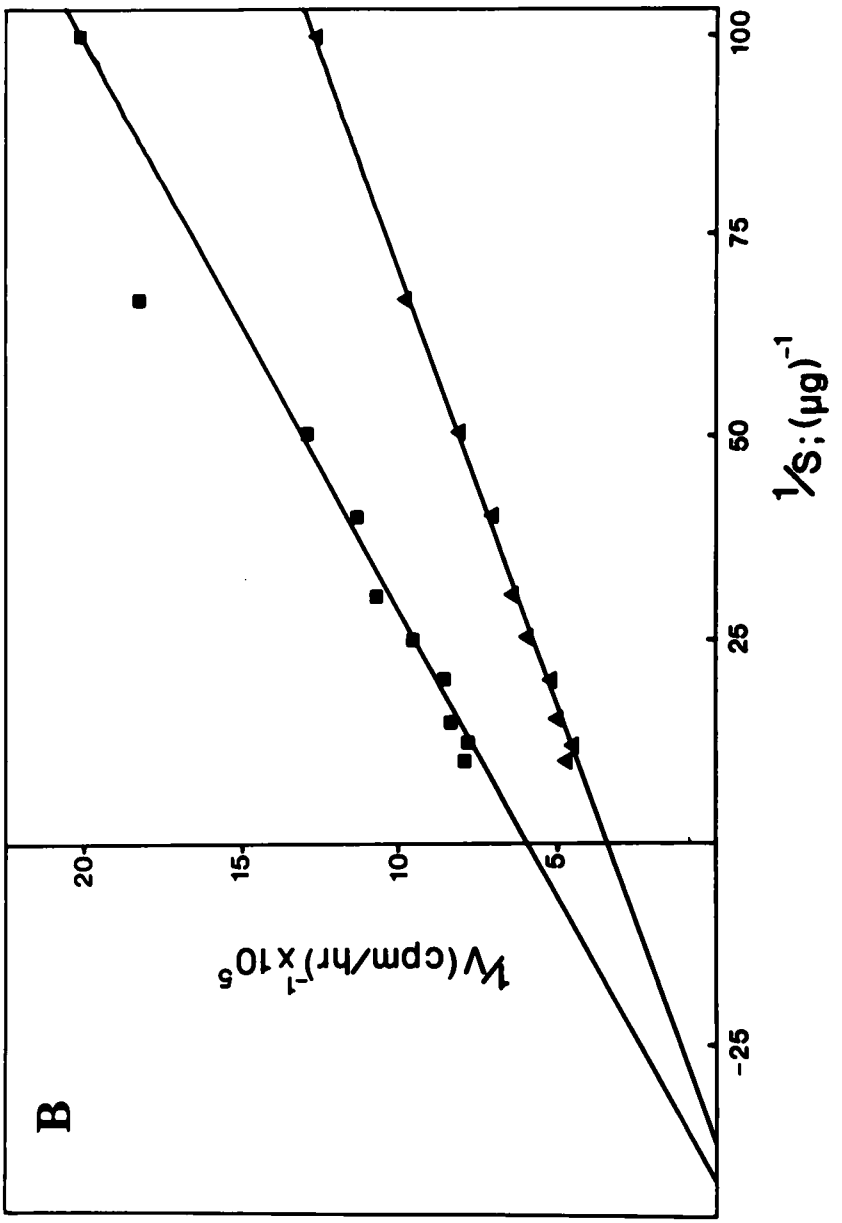

B
1/V (cpm/hr)$^{-1}$ x 10^5
20
15
10
5
-25
25
50
75
100
1/S; (µg)$^{-1}$

Table 1 Vmax values for 5'-flanking TNNCT mutants

Template		% change in Vmax
pV4a.5-45	$-38_{TCGCT-34}$	-
"	GCGCT	↓ 10.0%
pV4a.5-138	$-38_{TCGCT-34}$ (wild type)	-
"	GCGCT	↓ 28.2%
"	TCGAT	↓ 32.0%
"	TCGCA	↓ 32.8%
"	GCGGT	↓ 38.0%
"	GCGAT	↓ 42.0%
"	TCTCT	↓ 1.1%#
"	TAGCT	↑ 12.6%

Vmax was calculated from data obtained from ten or twelve point DNA input transcription experiments for each of the mutants and is expressed as percent increase or decrease over the value for pV4a.5-45 or pV4a.5-138 transcribed in parallel. Vmax values are the average of determinations using two different S-100 extracts except for #.

Figure 15 Autoradiogram of products from the transcription of pV4a.5-45 and pV4a.5-45,-38G

Autoradiograms representing the electrophoretic separation of labelled tRNAs from pV4a.5-45 and pV4a.5-45,-38 G are shown. Transcriptions were carried out using a total DNA concentration of 1.0 ug per reaction at 23.5° C and transcription products were analyzed on polyacrylamide gels as described in Materials and Methods. Lanes 1-12 show the transcription products of 3, 5, 7, 10, 15, 18, 25, 40, 50, 66.7, 100 and 150 ng of template DNA.

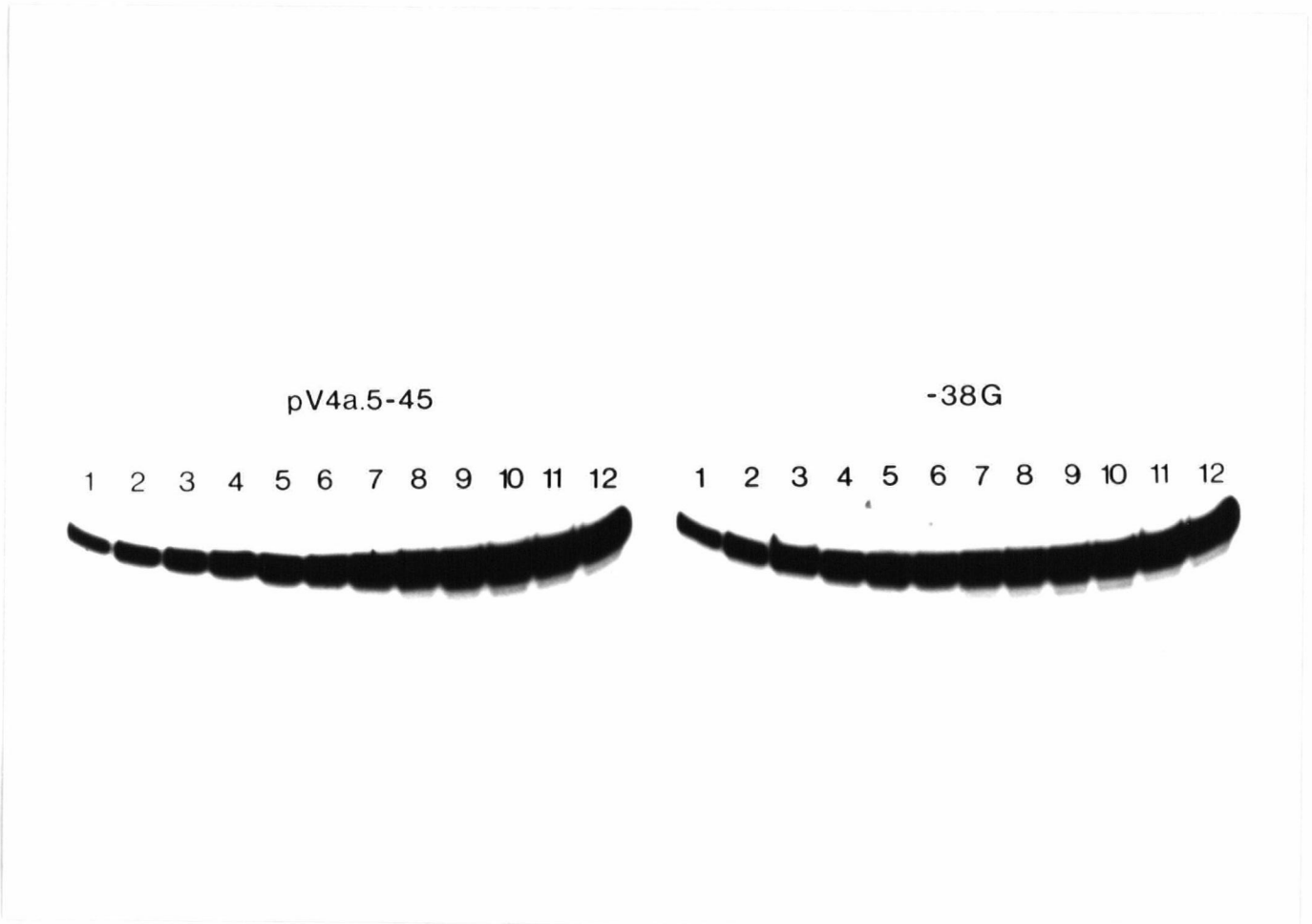

pV4a.5-45
1 2 3 4 5 6 7 8 9 10 11 12
-38G
1 2 3 4 5 6 7 8 9 10 11 12

Figure 16 Autoradiogram of products from the transcription of pV4a.5-138 and pV4a.5-138,-38G

Autoradiograms representing the electrophoretic separation of labelled tRNAs from pV4a.5-138 and pV4a.5-138,-38G are shown. Transcriptions were carried out using a total DNA concentration of 1.0 ug per reaction at 23.5° C and transcription products were analyzed on polyacrylamide gels as described in Materials and Methods. Lanes 1-10 show the transcription products of 5, 10, 15, 20, 25, 33, 40, 50, 66.7 and 100 ng of template DNA.

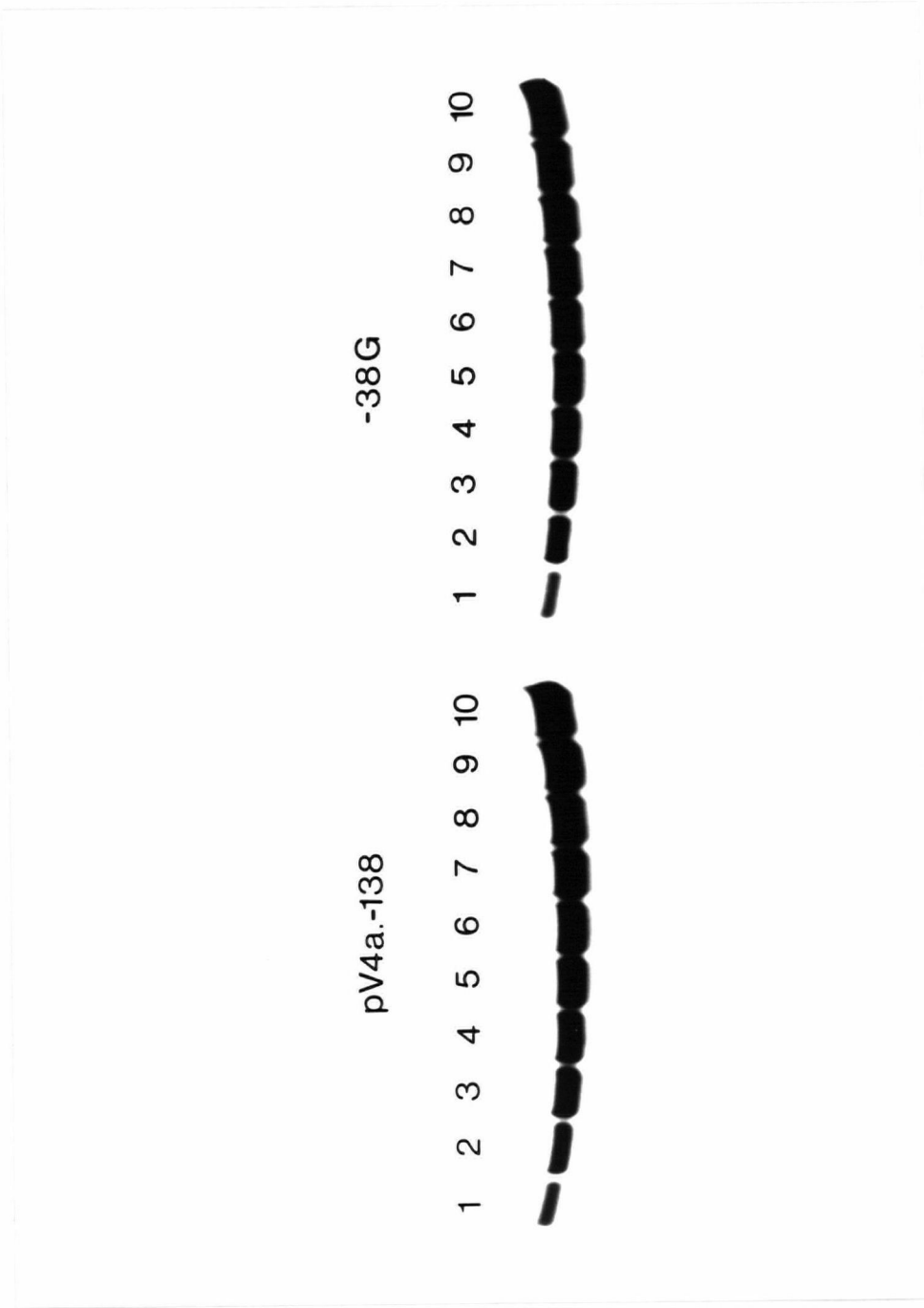

pV4a.-138
1 2 3 4 5 6 7 8 9 10
-38G
1 2 3 4 5 6 7 8 9 10

Figure 17 Autoradiogram of products from the transcription of pV4a.5-138 and pV4a.5-138,-35A

Autoradiograms representing the electrophoretic separation of labelled tRNAs from pV4a.5-138 and pV4a.5-138,-35A are shown. Transcriptions were carried out using a total DNA concentration of 1.0 ug per reaction at 23.5^{o} C and transcription products were analyzed on polyacrylamide gels as described in Materials and Methods. Lanes 1-10 show the transcription products of 10, 15, 20, 25, 33, 40, 50, 66.7, 84 and 100 ng of template DNA.

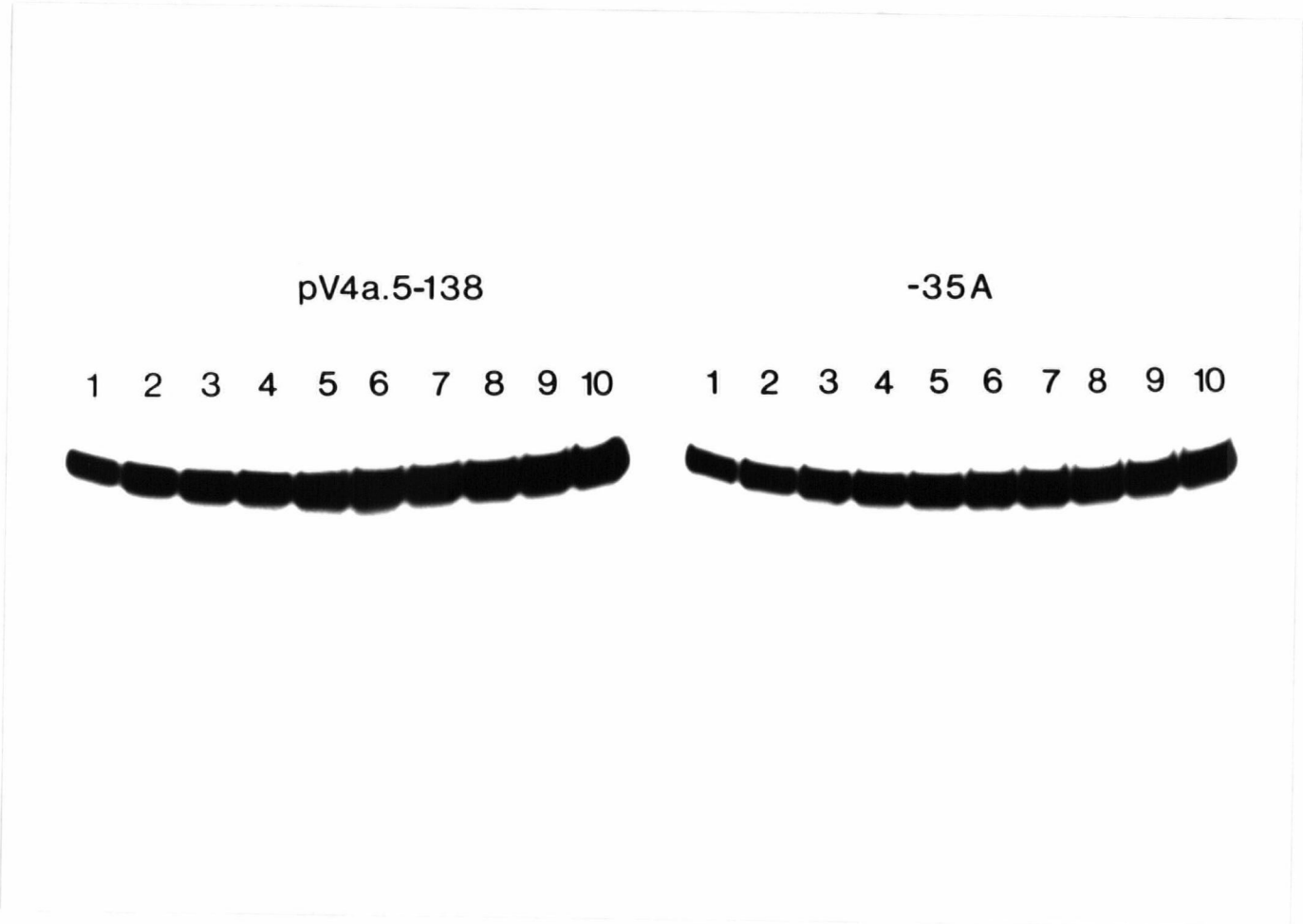

pV4a.5-138
1 2 3 4 5 6 7 8 9 10
-35A
1 2 3 4 5 6 7 8 9 10

Figure 18 Autoradiogram of products from the transcription of pV4a.5-138 and pV4a.5-138,-34A

Autoradiograms representing the electrophoretic separation of labelled tRNAs from pV4a.5-138 and pV4a.5-138,-34A are shown. Transcriptions were carried out using a total DNA concentration of 1.0 ug per reaction at 23.5° C and transcription products were analyzed on polyacrylamide gels as described in Materials and Methods. Lanes 1-10 show the transcription products of 10, 15, 20, 25, 33, 40, 50, 66.7, 84 and 100 ng of template DNA.

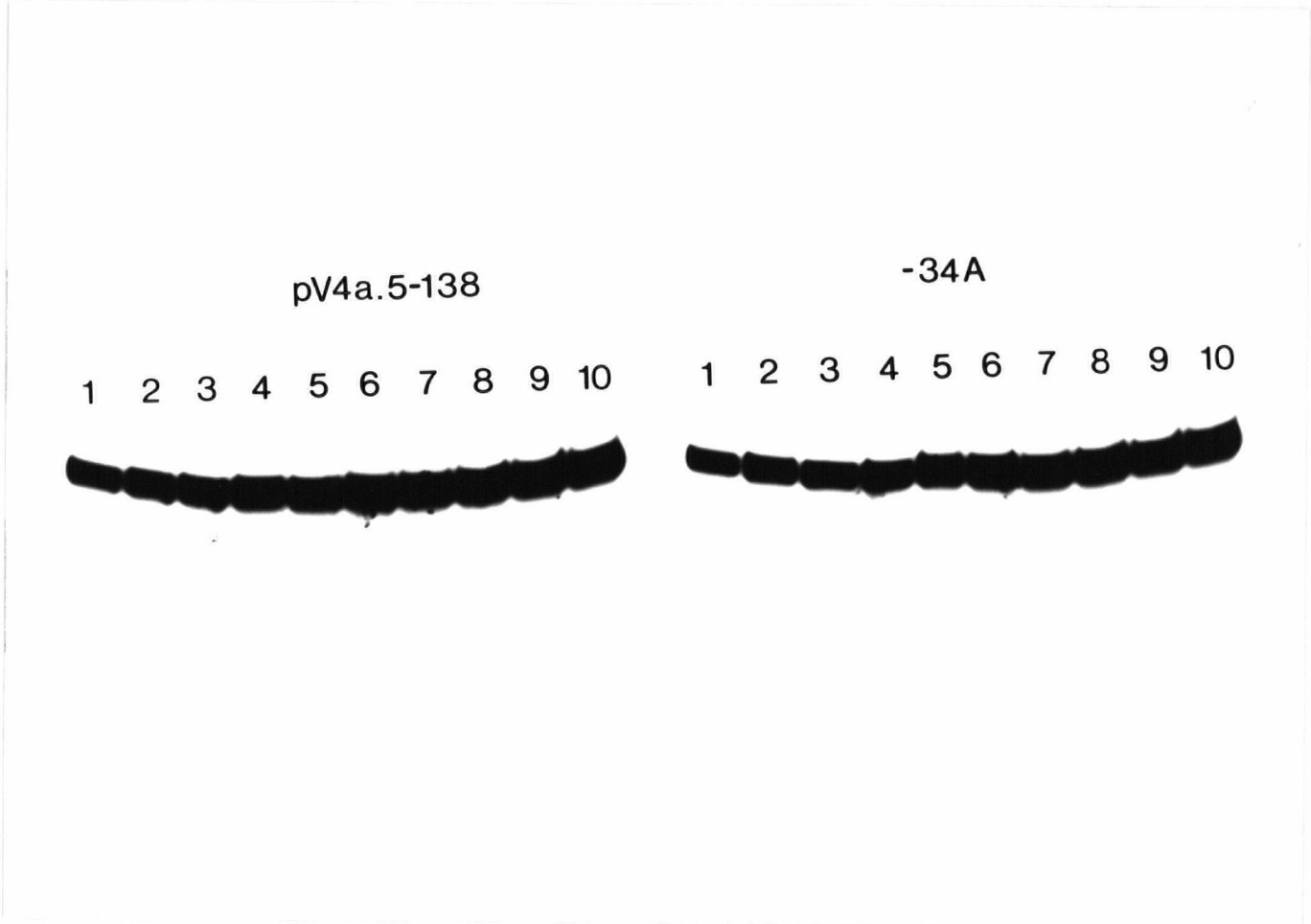

pV4a.5-138
1 2 3 4 5 6 7 8 9 10
-34A
1 2 3 4 5 6 7 8 9 10

Figure 19 Autoradiogram of products from the transcription of pV4a.5-138 and pV4a.5-138,-38G,-35G

Autoradiograms representing the electrophoretic separation of labelled tRNAs from pV4a.5-138 and pV4a.5-138,-38G,-35G are shown. Transcriptions were carried out using a total DNA concentration of 1.0 ug per reaction at 23.5° C and transcription products were analyzed on polyacrylamide gels as described in Materials and Methods. Lanes 1-10 show the transcription products of 10, 15, 20, 25, 33, 40, 50, 66.7, 84 and 100 ng of template DNA.

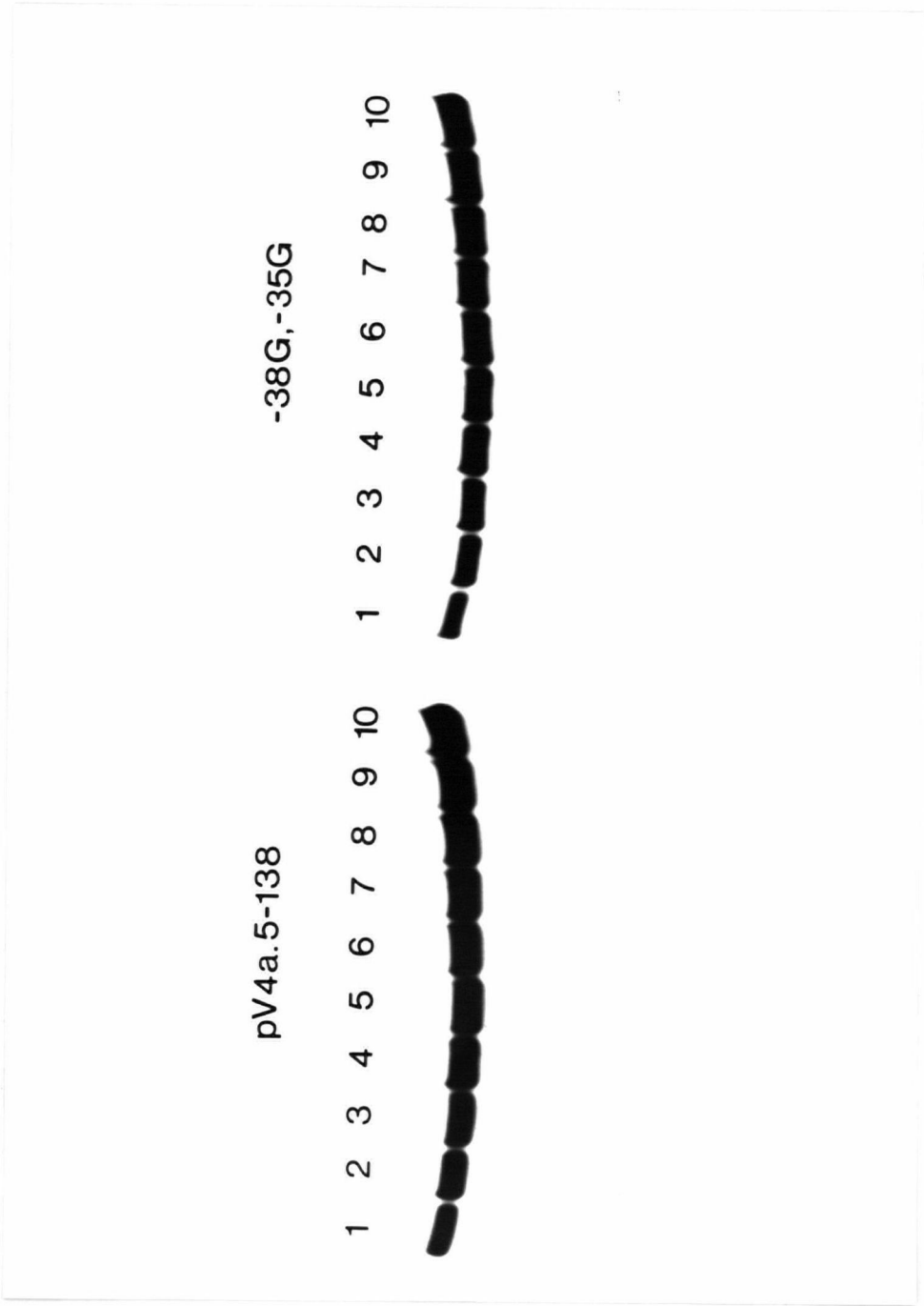

pV4a.5-138
1 2 3 4 5 6 7 8 9 10
-38G,-35G
1 2 3 4 5 6 7 8 9 10

Figure 20 Autoradiogram of products from the transcription of pV4a.5-138 and pV4a.5-138,-36T

Autoradiograms representing the electrophoretic separation of labelled tRNAs from pV4a.5-138 and pV4a.5-138,-36T are shown. Transcriptions were carried out using a total DNA concentration of 1.0 ug per reaction at 23.5° C and transcription products were analyzed on polyacrylamide gels as described in Materials and Methods. Lanes 1-10 show the transcription products of 10, 15, 20, 25, 33, 40, 50, 66.7, 84 and 100 ng of template DNA.


pV4a.5-138
1 2 3 4 5 6 7 8 9 10
-36T
1 2 3 4 5 6 7 8 9 10

Vmax of 38% and 42% (Table 1;Figures 19 and 14A). However, a mutation at position N36 led to only a 1.1% decrease in Vmax (Figure 20). The nucleotide change at position N37 resulted in a 12.6% increase in Vmax relative to pV4a.5-138 (Figure 21). Therefore the results showed a sequence of the form TNNCT to be responsible for the modulation of transcription in pV4a.5-138, but the mechanism of this modulation remained to be determined.

B. Internal TNNCT mutants

The $tRNA^{Val}_4$ clone contained TNNCT sequences in the 5'-flanking region as well as in the mature coding sequence (Figure 13). Since TNNCT was found to modulate transcription from the 5'-flanking region of the gene, the TNNCT in the stem/loop region of pV4a.5-138 was also mutated to determine its effect on transcription. The template containing the internal TNNCT mutant was found to transcribe at a 46.4% lower efficiency than pV4a.5-138 (Table 2). This may have been in part due to the abundance of what appeared to be partial transcription products on the gel (Figure 22) since only the mature length transcripts were quantitated by Cerenkov counting. The template mutant in both internal and 5'-flanking TNNCTs had a transcription efficiency 54.3% lower than pV4a.5-138 (Table 2). Although partial transcripts were also present for the double TNNCT mutant (Figure 23), the lowered activity did not allow their easy detection by autoradiography.

Figure 21 Autoradiogram of products from the transcription of pV4a.5-138 and pV4a.5-138,-37A

Autoradiograms representing the electrophoretic separation of labelled tRNAs from pV4a.5-138 and pV4a.5-138,-37A are shown. Transcriptions were carried out using a total DNA concentration of 1.0 ug per reaction at 23.5° C and transcription products were analyzed on polyacrylamide gels as described in Materials and Methods. Lanes 1-10 represent the transcription products of 10, 15, 20, 25, 33, 40, 50, 66.7, 84 and 100 ng of template DNA.

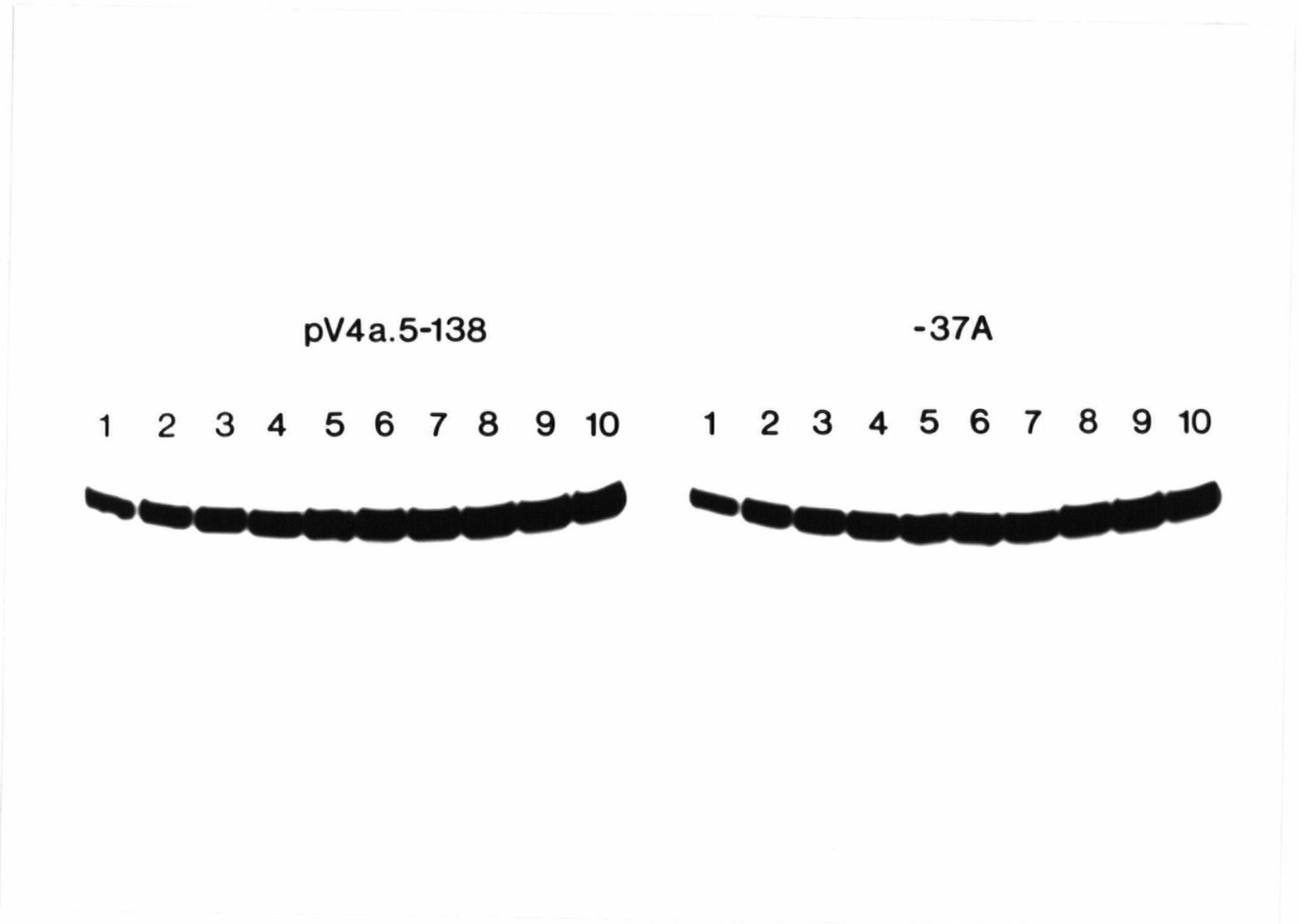

pV4a.5-138
-37A
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10

Figure 22 Autoradiogram of products from the transcription of pV4a.5-138 and pV4a.5-138,+29A,+32G,+33C

Autoradiograms representing the electrophoretic separation of labelled tRNAs from pV4a.5-138 and pV4a.5-138,+29A,+32G,+33C are shown. Transcriptions were carried out using a total DNA concentration of 1.0 ug per reaction at 23.5^{o} C and transcription products were analyzed on polyacrylamide gels as described in Materials and Methods. Lanes 1-10 represent the transcription products of 10, 15, 20, 25, 33, 40, 50, 66.7, 84 and 100 ng of template DNA.

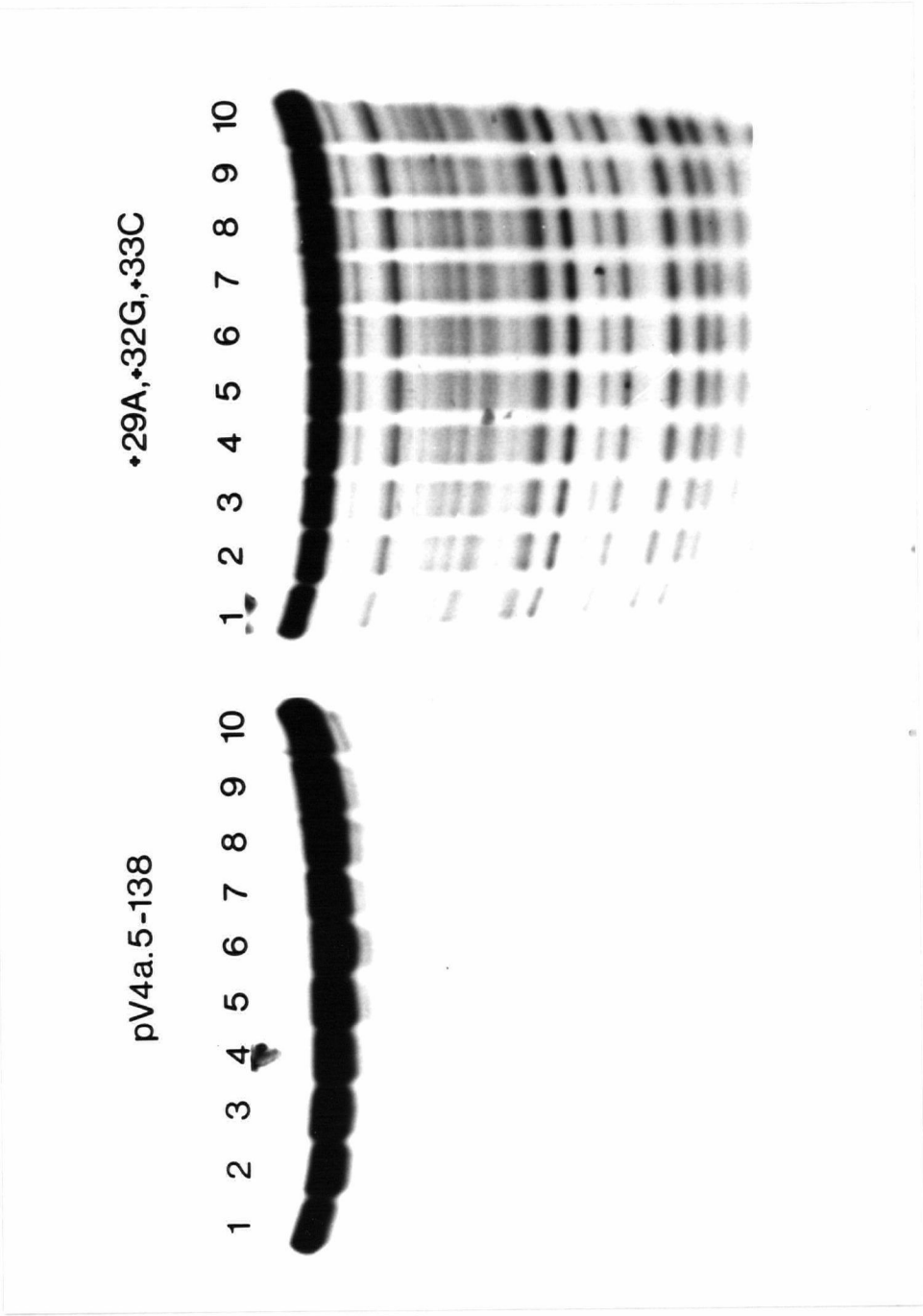

pV4a.5-138
1 2 3 4 5 6 7 8 9 10
+29A,+32G,+33C
1 2 3 4 5 6 7 8 9 10

Table 2 Vmax values for internal TNNCT mutants

Template			% change in Vmax
pV4a.5-138	$^{-38}$TCGCT^{-34}	$^{+29}$TGCCT^{+33}	-
"	"	AGCGC	↓46.4%
"	GCGAT	AGCGC	↓54.3%

Vmax was calculated from data obtained from ten point DNA input transcription experiments for each of the mutants and is expressed as percent increase or decrease over the value for pV4a.5-138 transcribed in parallel.

Figure 23 Autoradiogram of products from the transcription of pV4a.5-138 and pV4a.5-138,-38G,-35A,+29A,+32G +33C

Autoradiograms representing the electrophoretic separation of labelled tRNAs from pV4a.5-138 and pV4a.5-138,-38G, -35A, +29A, +32G, +33C are shown. Transcriptions were carried out using a total DNA concentration of 1.0 ug per reaction at 23.5° C and transcription products were analyzed on polyacrylamide gels as described in Materials and Methods. Lanes 1-10 represent the transcription products of 10, 15, 20, 25, 33, 40, 50, 66.7, 84 and 100 ng of template DNA.

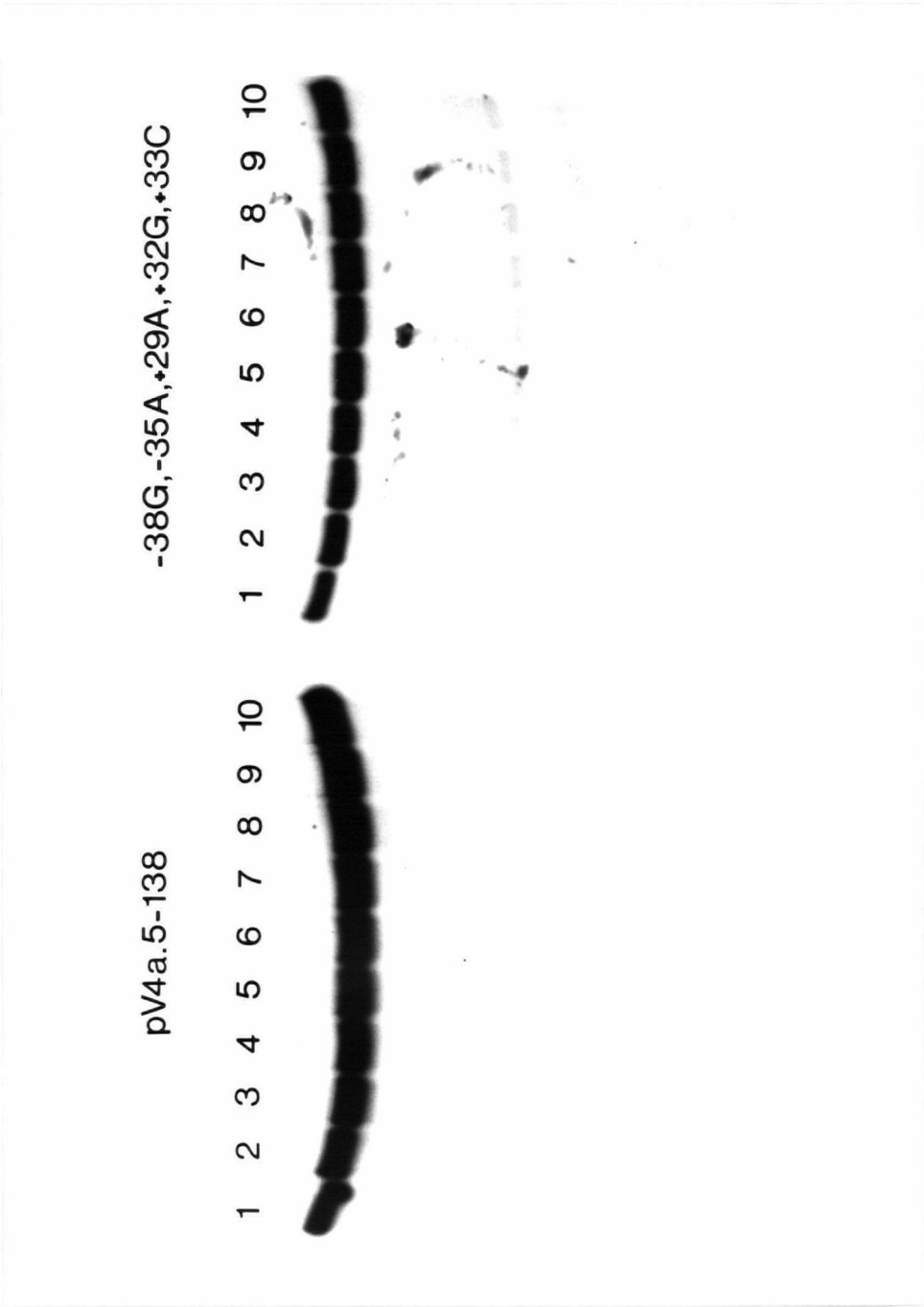

pV4a.5-138
1 2 3 4 5 6 7 8 9 10
-38G,-35A,+29A,+32G,+33C
1 2 3 4 5 6 7 8 9 10

Figure 24 The sequence of pS7a.

Sequence of the non-coding strand of pS7a. is shown to position -130. The 3'-flanking region following the poly T termination signal is not shown. The sequence AGTTG is underlined and the internal TNNCTs are double underlined. The first mature nucleotide of the $tRNA^{Ser}_{7}$ gene and the D and T control sequences are in bold letters.

$^{-130}$CTTGGCGCTCAAATTCAAGTAACACACACATGCAGTGTTGTCAAATGAGCA^{-80}

$^{-79}$AGGTTCCGAAATGTGTGTTCAGTCTTGGATTCTCCCATGCAATCAACATTA^{-29}

$^{-28}$GTTGCCAATTTGCCGTGTCATACCAACAGCAGTCGTGGCCGAGCGGTTAAG^{+23}

$^{+22}$GCGTCTGACTAGAAATCAGATTCCCTCTGGGAGCGTAGGTTCGAATCCTAC^{+74}

$^{+75}$CGACTGCGAGAAGGTTTACGGATTTTTTTTATTTTT^{+110}

C. Deletion mutants

In contrast to the $tRNA^{Val}_4$ gene which appeared to modulate its transcription by the sequence TNNCT, the $tRNA^{Ser}_7$ gene contained in pS7a.5-125 (Figure 24) directed transcription in the absence of a TNNCT in the 5'-flanking region. Therefore a number of deletion mutants were created in pS7a.5-125 to define the sequences responsible for its efficient transcription. For transcription of the 5'-flanking deletion series in pS7a.5-125, the values for Vmax were expressed as the percent increase or decrease of the Vmax value for pS7a.5-119 (Table 3). Deletion of sequences to position -31 did not reduce the level of transcription and resulted in a small increase (8.2%) in Vmax (Figure 25). Deletion of an additional seven nucleotides to position -24 relative to the mature coding sequence reduced the Vmax 57.1% relative to the control (Figure 26). Deletion end-point -24 resulted in the removal of the sequence $^{-29}$AGTTG^{-25} which was also present in the 5'-flank of pV4a.5-138 and a $tRNA^{Arg}$ gene. Further deletion of 5'-flanking sequences to position -18 severely decreased the level of transcription (Table 3) (81.5% decrease in Vmax) as shown in Figure 27.

Table 3 Vmax values for 5'-flanking deletion mutants of pS7a.5-119

Template	% change in Vmax
pS7a.5-119	-
pS7a.5-31	↑ 8.2%
pS7a.5-24	↓ 57.1%
pS7a.5-18	↓ 81.5%

Vmax was calculated from data obtained from ten point DNA input transcription experiments for each of the mutants and is expressed as percent increase or decrease over the value for pS7a.5-119 transcribed on parallel.

Figure 25 Autoradiogram of products from the transcription of pS7a.5-119 and pS7a.5-31

Autoradiograms representing the electrophoretic separation of labelled tRNAs from pS7a.5-119 and pS7a.5-31 are shown. Transcriptions were carried out using a total DNA concentration of 1.0 ug per reaction at 23.5° C and transcription products were analyzed on polyacrylamide gels as described in Materials and Methods. Lanes 1-10 represent the transcription products of 10, 15, 20, 25, 33, 40, 50, 66.7, 84 and 100 ng of template DNA.

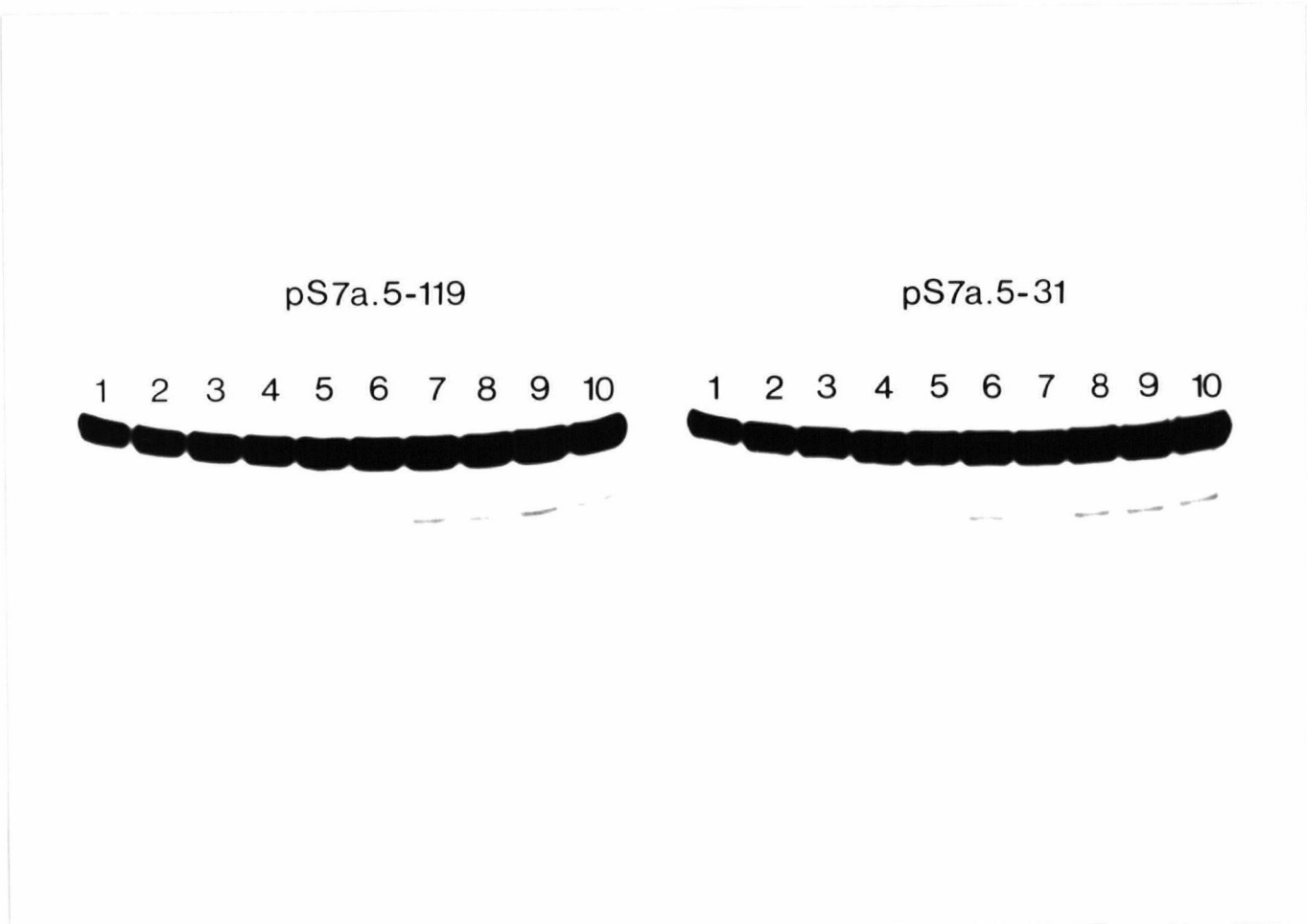

pS7a.5-119
1 2 3 4 5 6 7 8 9 10
pS7a.5-31
1 2 3 4 5 6 7 8 9 10

Figure 26 Autoradiogram of products from the transcription of pS7a.5-119 and pS7a.5-24

Autoradiograms representing the electrophoretic separation of labelled tRNAs from pS7a.5-119 and pS7a.5-24 are shown. Transcriptions were carried out using a total DNA concentration of 1.0 ug per reaction at 23.5° C and transcription products were analyzed on polyacrylamide gels as described in Materials and Methods. Lanes 1-10 represent the transcription products of 10, 15, 20, 25, 33, 40, 50, 66.7, 84 and 100 ng of template DNA.

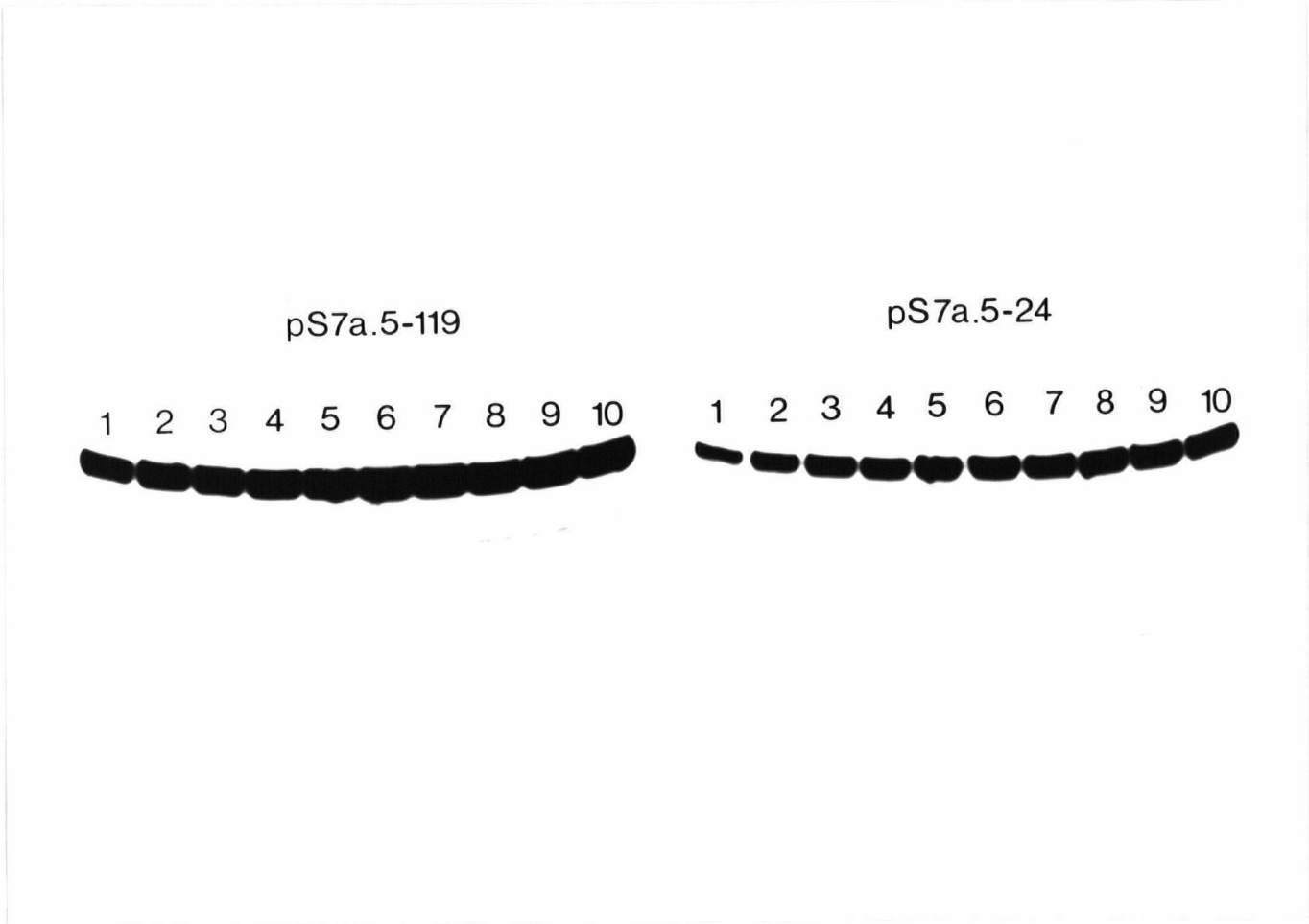
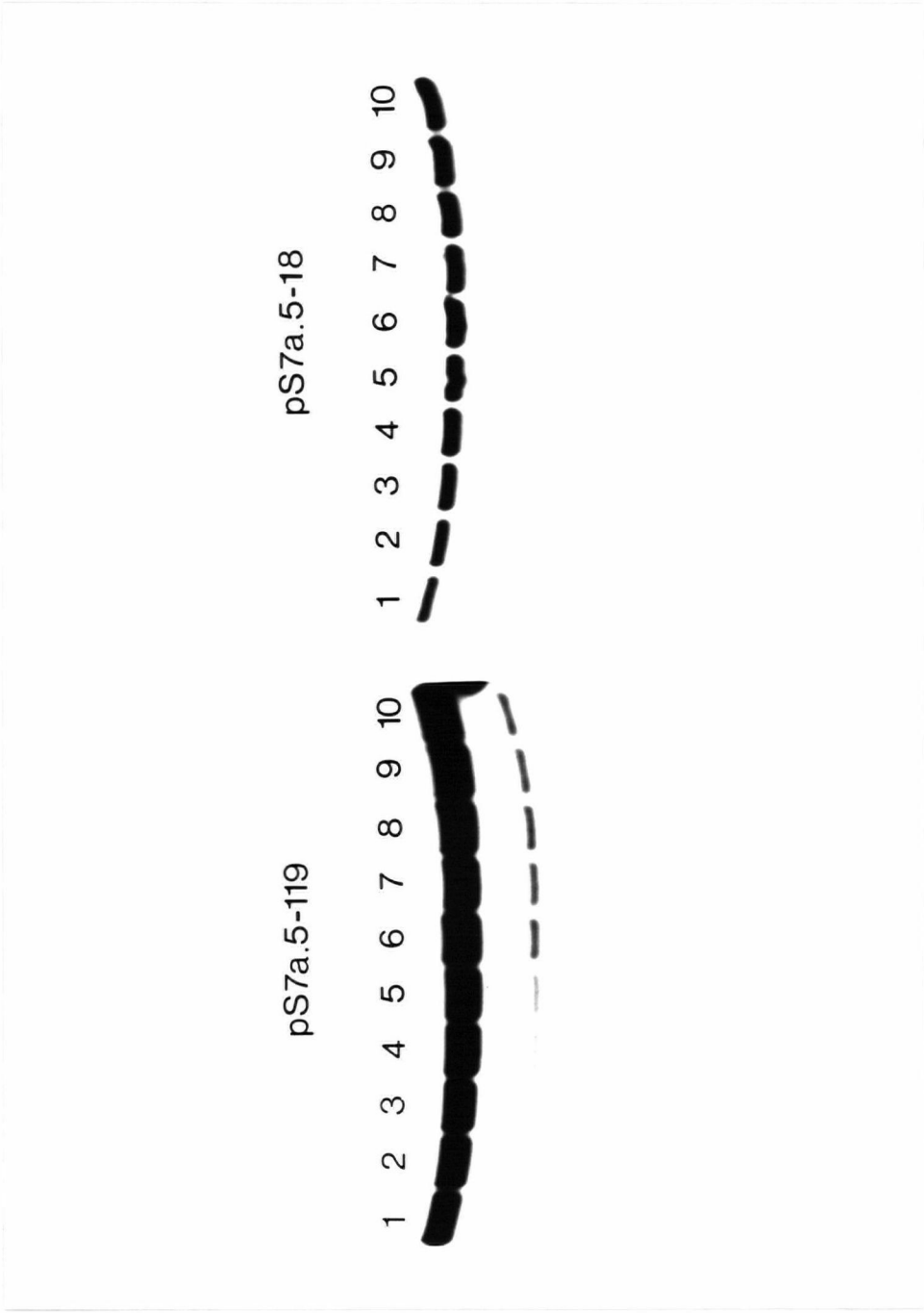

pS7a.5-119
1 2 3 4 5 6 7 8 9 10
pS7a.5-24
1 2 3 4 5 6 7 8 9 10

Figure 27 Autoradiogram of products from the transcription of pS7a.5-119 and pS7a.5-18

Autoradiograms representing the electrophoretic separation of labelled tRNAs from pS7a.5-119 and pS7a.5-18 are shown. Transcriptions were carried out using a total DNA concentration of 1.0 ug per reaction at 23.5° C and transcription products were analyzed on polyacrylamide gels as described in Materials and Methods. Lanes 1-10 represent the transcription products of 10, 15, 20, 25, 33, 40, 50, 66.7, 84 and 100 ng of template DNA.


pS7a.5-18
1 2 3 4 5 6 7 8 9 10
pS7a.5-119
1 2 3 4 5 6 7 8 9 10

IV. Gel retardation assay and transcription competition with isolated 5'-flank of pV4a.5-179

In an attempt to define TNNCT function and its importance in directing efficient transcription, the following experiments were carried out.

A. Gel retardation

Gel retardation has been used as a tool for detection of protein factors which bind specifically to sequences involved in promoter and enhancer function of various viral and eukaryotic genes (Kovesdi et al., 1986; Jones et al., 1987). Since one possible mechanism for the function of modulatory sequences of tRNA genes could involve the interaction of transcription factors with 5'-flanking sequences, a gel retardation assay was used to detect binding to the isolated 5'-flank of pV4a.5-179. To determine whether transcription factor(s) interacted with the 5'-flank of the $tRNA^{Val}_{4}$ gene, the HindIII fragment containing 179 bp of 5'-flanking sequence in pEMBL8- was isolated and labelled with dATP using Klenow polymerase at both ends as described in Materials and Methods. The labelled probe was then used in a gel retardation assay as outlined in Materials and Methods.

Figure 28 shows an autoradiogram of the gel of pV4a.5-179 5'-flank used as probe after incubation with increasing amounts of S-100 extract. The control in this experiment was the labelled 5'-flank (lane 1, Figure 28) which migrated alongside the unbound probe in reactions containing S-100

Figure 28 Gel retardation assay

The figure shows gel retardation of labelled probe (179 bp Val_4 5'-flank) with increasing amounts of S-100 extract. Each lane contains 30,000 cpm of 5'-flank.

Lanes 1-8 contain 1.56 ug of pUC13 DNA as competitor

Lanes 8-14 contain 3 ug of poly dI-dC DNA as competitor

Lane 1 = no S-100

Lanes 2&9 = 0.05 ul S-100

Lanes 3&10 = 0.1 ul S-100

Lanes 4&11 = 0.3 ul S-100

Lanes 5&12 = 0.5 ul S-100

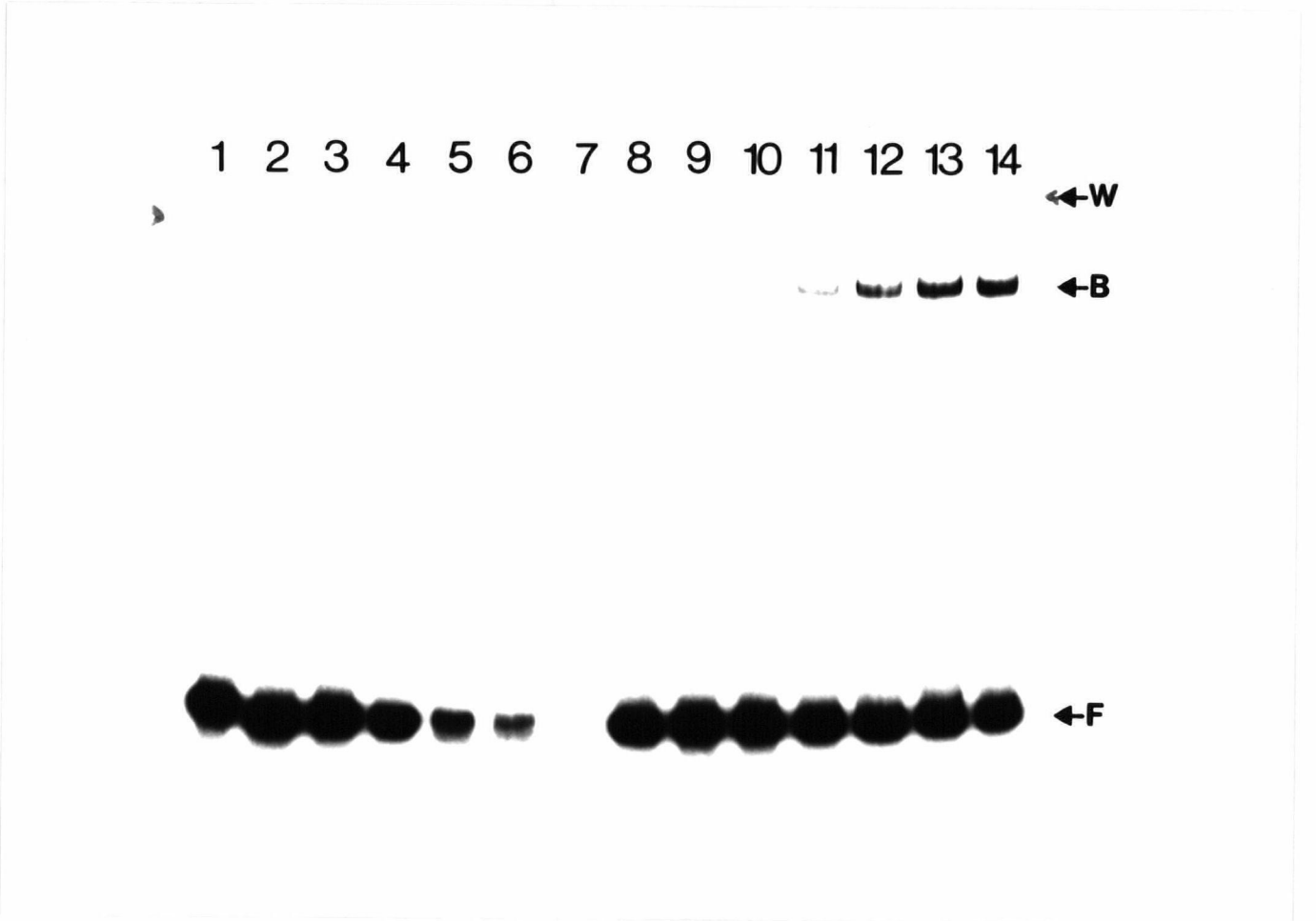
Lanes 6&13 = 0.7 ul S-100

Lanes 7&14 = 0.9 ul S-100

Lane 8 = 0.9 ul S-100, with both pUC 13 and poly dI-dC DNA

Binding reactions were performed as described in Materials and Methods. Products were electrophoresed on a 4% polyacrylamide gel containing TGE. The gel was dried prior to autoradiography.

F = free probe; B = bound probe; W = position of well


1 2 3 4 5 6 7 8 9 10 11 12 13 14
W
B
F

extract. Previous results had shown that no retardation was detected when the probe was incubated in the extract without the addition of non-specific competitor DNA (data not shown). In this experiment, protein interaction was not detected when pUC DNA (normally required for efficient transcription *in vitro*) was used as competitor DNA (Figure 28, lanes 2-7). However, a single retarded band was observed when poly dI-dC was used as non-specific competitor DNA (Figure 28, lanes 9-14). As little as 0.1 ul of S-100 was required to detect binding activity.

It became evident that a nuclease activity was present in the S-100 extract which resulted in the degradation of labelled probe, as seen in lanes 2-7 in Figure 28. This nuclease activity was diminished when poly dI-dC was used as non-specific DNA in the assay. However, when increasing amounts of extract were added to reactions containing poly dI-dC, more free probe became degraded due to increasing levels of nuclease activity (lanes 9-14, Figure 28). When both poly dI-dC and pUC DNA were present (Figure 28, lane 8), the free probe was protected, but no retarded band was observed. Therefore it appears that pUC DNA competes very successfully for the protein which, in its absence, binds to the labelled 5'-flank of the tRNA template. On the other hand, poly dI-dC did not compete with the template for the S-100 transcription factor(s). In a separate experiment binding of protein was still detectable even when up to 30

ug of poly dI-dC was included in an assay containing 0.7 ul of S-100 extract (data not shown).

B. Transcription competition between the $tRNA^{Val}_4$ 5'-flank and pV4a.5-138

It was reasoned that if the factor binding to the 5'-flank of pV4a.5-179 was required for directing efficient transcription, then it might be possible to remove the transcription factor(s) from the template containing a tRNA gene by the addition of increasing amounts of plasmid DNA containing the $tRNA^{Val}_4$ 5'-flank. The pV4a.5-179 5'-flank in pEMBL8- was used as competitor for the transcription of 0.01 ug of pV4a.5-138. Competition experiments were carried out by the addition of 0 to 1.2 ug of competitor DNA either simultaneously or to preformed (40 min) stable complexes (Figure 29, lanes 7-10). All transcription reactions contained pUC13 DNA to a total of 1.2 ug per reaction except for the reaction containing 1.2 ug of 5'-flank (lane 6, Figure 29). Reactions were carried out for 90 min before termination. Products were analyzed on a polyacrylamide gel and following autoradiography, the amount of product from each reaction was determined as described in Materials and Methods.

Results indicated no change in the level of pV4a.5-138 transcription regardless of whether competitor DNA was added simultaneously with the pV4a.5-138 gene or after preformed complexes of pV4a.5-138. Therefore the $tRNA^{Val}_4$ 5'-flank was unable to compete for factor(s) in $tRNA^{Val}_4$

Figure 29 Transcription competition between the Val_4 5'-flank and pV4a.5-138

The autoradiogram shows the electrophoretic separation of labelled tRNAs from pV4a.5-138 used in a competition assay with the Val_4 5'-flank contained in pEMBL8-. All lanes contained 0.01 ug of pV4a.5-138 serving as template DNA.

Lanes 1&7 = 0 ug competitor DNA (Val_4 5'-flank)

" 2&8 = 0.4 " " "

" 3&9 = 0.6 " " "

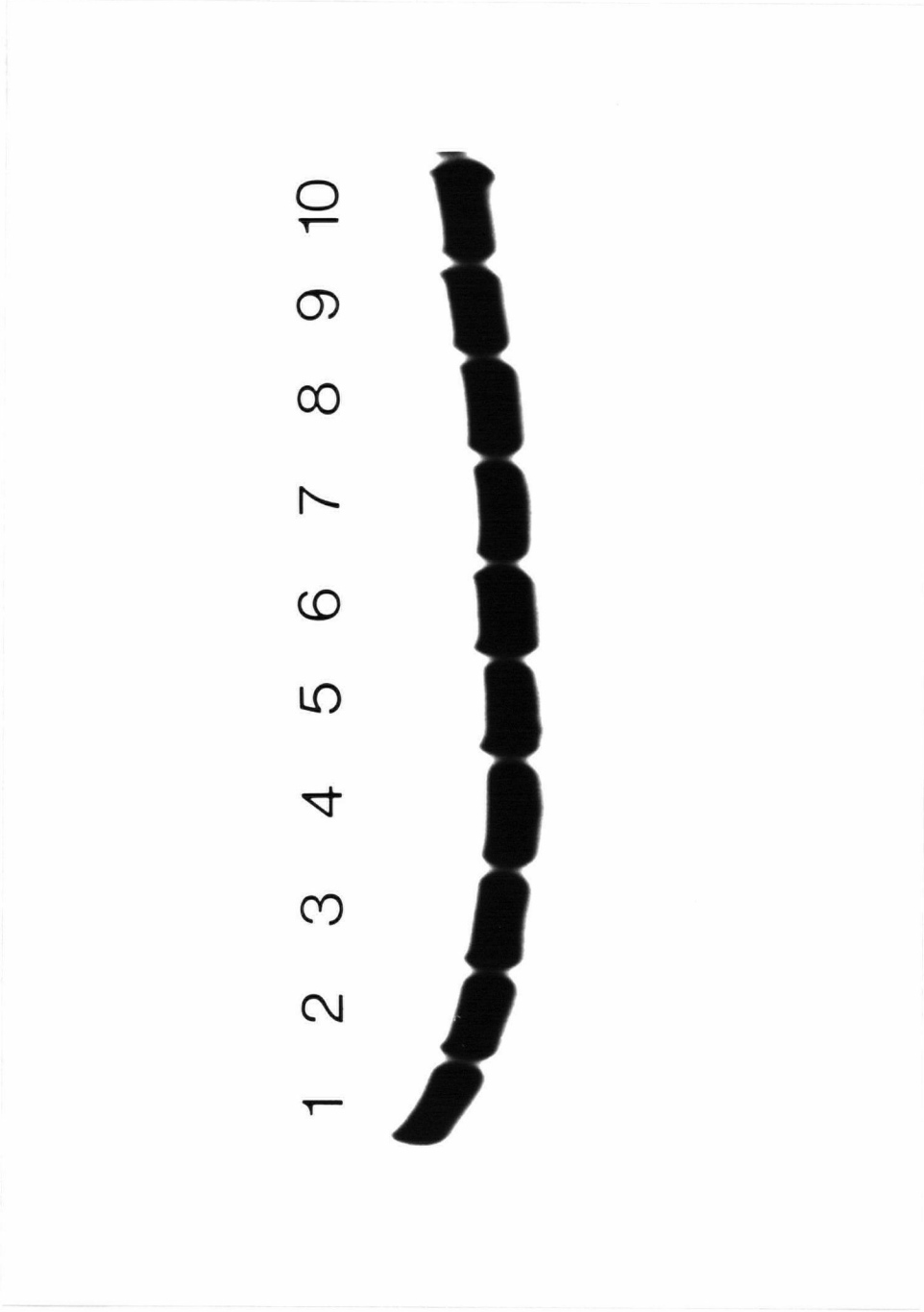
" 4&10 = 0.8 " " "

" 5&6 = 1.0 and 1.2 ug competitor DNA respectively

" 2-6 = competitor and pV4a.5-138 DNAs added simultaneously

" 8-10 = competitor DNA added following the formation of a 40 min stable complex of pV4a.5-138.

All reactions contained pUC13 DNA to a total of 1.2 ug per reaction, except for lane 6, to which no pUC DNA was added.


1 2 3 4 5 6 7 8 9 10

transcription, when isolated from its gene. Results obtained from the gel retardation experiment (section A) and from the transcription competition suggested that while the S-100 extract contained a protein capable of binding to the 5'-flank of pV4a.5-179 and pUC DNA, the factor did not appear to be required for transcription. Therefore, it was unlikely that the 5'-flank sequestered transcription factor(s) essential for efficient transcription, at least in the absence of D and T control regions. It may be that pUC DNA had a greater affinity for the factor than the isolated 5'-flank. However, the pUC DNA was absent in one of the transcription competition reactions (lane 6, Figure 29) and in this reaction the 5'-flank of the $tRNA^{Val}_4$ gene completely lacked competitive ability, which indicated that it was not binding a factor required for transcription.

V. Transcription competition between pV4a.5-138,-38G,-35A and pS7a.5-119

Competition experiments have previously been used to compare the the ability of various templates in binding transcription factors for stable complex formation and in the efficiency of directing transcription (Dingermann et al., 1983; Johnson-Burke et al., 1983; Fuhrman et al., 1984; Johnson-Burke and Soll, 1985; Lofquist and Sharp, 1986). The following experiments were carried out to determine the efficiency of complex formation for the template mutant in 5'-flanking TNNCT.

A. Simultaneous addition

To determine the relative competitive strength of the templates mutant in 5'-flanking TNNCT in transcription, the pV4a.5-138,-38G,-35A mutant was used as competitor DNA against pS7a.5-119. The $tRNA^{Ser}_{7}$ and $tRNA^{Val}_{4}$ genes were comparable in transcription efficiency (St. Louis and Spiegelman, 1985) and since the $tRNA^{Ser}_{7}$ gene transcript is longer in length than the $tRNA^{Val}_{4}$ gene transcript, the two could be effectively separated on a polyacrylamide gel. Thus the products of both template DNAs in the competition assay could be separated and quantified. The control in this experiment was pV4a.5-138 (wild type).

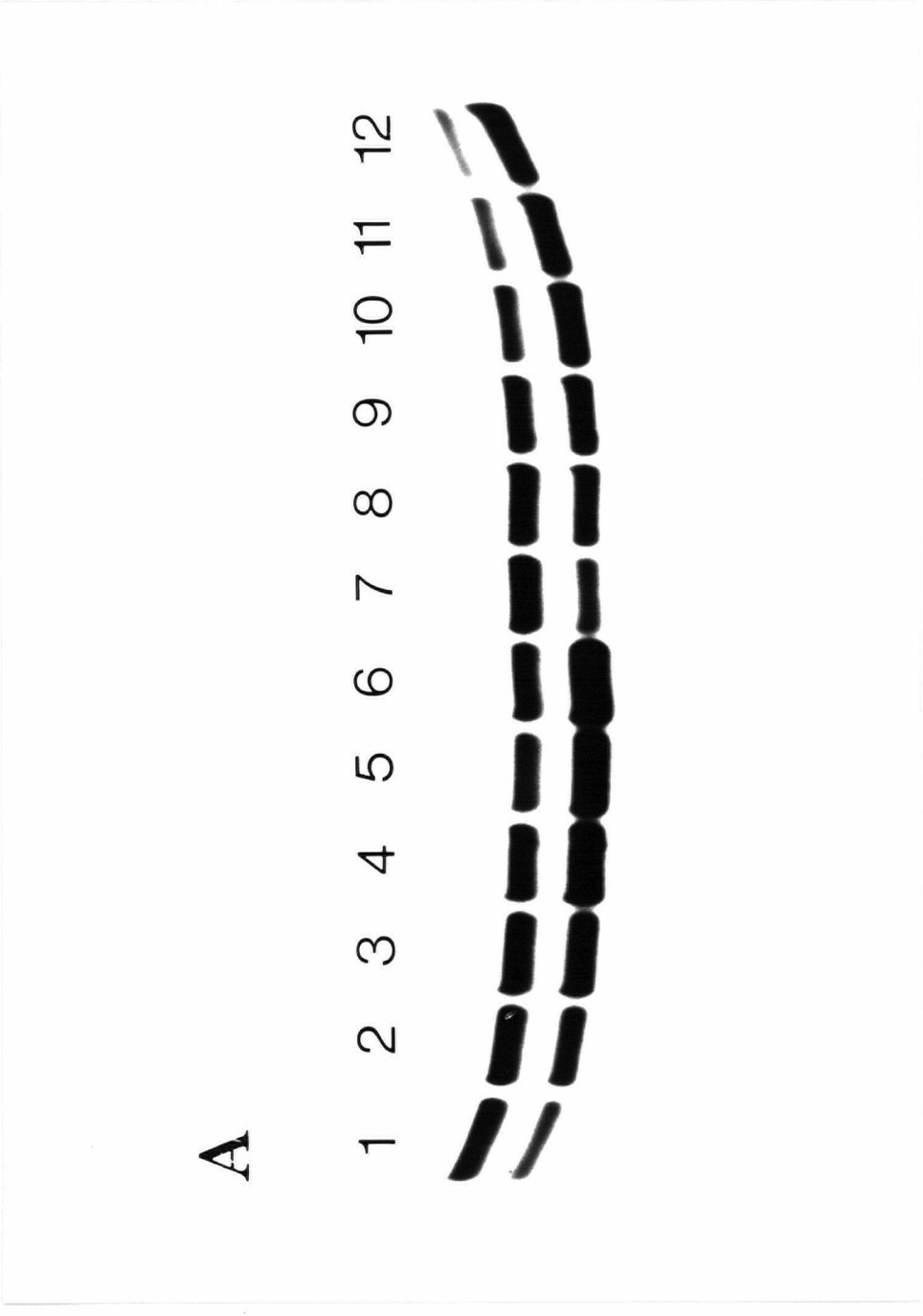
Transcriptions from six different concentrations of template DNA (10-50 ng) were carried out simultaneously by premixing varying amounts of pV4a.5-138, or pV4a.5-138, -38G,-35A DNAs with 10 ng of pS7a.5-119 prior to the addition of S-100 extract. Reactions were carried out for 90 min and products were analyzed as described in Materials and Methods (Figure 30-A). The amount of product for each reaction was quantified and the data plotted (Figure 30-B) in two ways. When data from the velocity of transcriptions of pV4a.5-138 wild type and mutant were graphed as a function of DNA input, the results showed no apparent effects on their relative transcription rates (i.e. the wild type $tRNA^{Val}_{4}$ gene still displayed a higher Vmax than the 5'-flanking TNNCT mutant). However, when the data from the transcriptions of pS7a.5-119 were plotted, the mutant

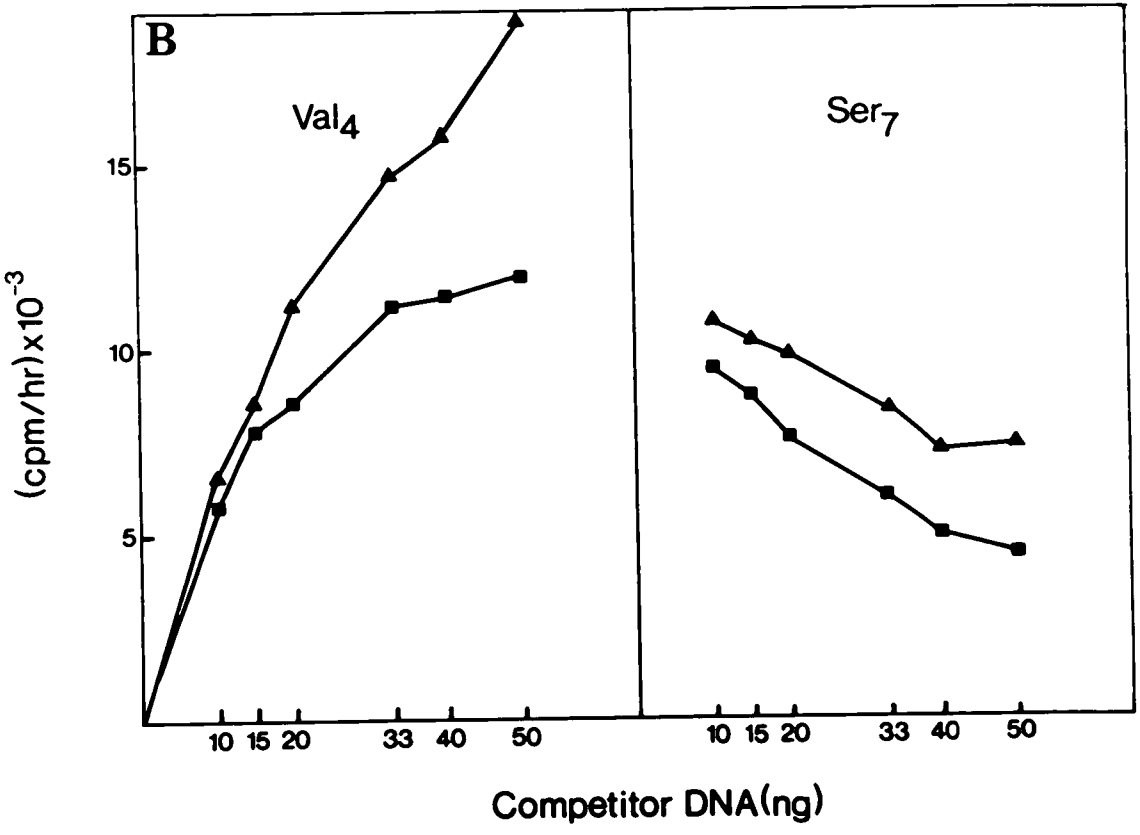
Figure 30-A Transcription competition between pV4a.5-138, -38G,-35A and pS7a.5-119

The autoradiogram shows the electrophoretic separation of labelled tRNAs from pV4a.5-138 wild type (lanes 1-6, lower band) and pV4a.5-138,-38G,-35A (lanes 7-12, lower band) used in a competition experiment with pS7a.5-119 (lanes 1-12, upper band). All lanes contained 10 ng of pS7a.5-119 and pUC13 DNA to a total of 1.0 ug. Transcriptions were carried out at 23.5° C and transcription products were analyzed on a 10% polyacrylamide gel as described in Materials and Methods. Lanes 1-6 and 7-12 represent the transcription products of 10, 15, 20, 33, 40 and 50 ng of pV4a.5-138 and pV4a.5-138,-38G,-35A competitor dNAs respectively.

B Graph of data from transcription competition between pV4a.5-138 wild type (▲), pV4a.5-138,-38G,-35A (■) and pS7a.5-119

Gel slices corresponding to transcription products were excised and the amount of product determined by Cerenkov counting. The data were plotted as cpm of transcript per hour of reaction versus competitor template DNA input. The left panel shows transcription from pV4a.5-138 wild type (▲) and pV4a.5-138,-38G,-35A (■). The right panel shows transcription of pS7a.5-119 in the presence of pV4a.5-138 (▲) and pV4a.5-138,-38G,-35A (■).


A
1 2 3 4 5 6 7 8 9 10 11 12


B
Val4
Ser7
15
10
5
(cpm/hr)x10-3
10 15 20 33 40 50
10 15 20 33 40 50
Competitor DNA(ng)

pV4a.5-138,-38G,-35A was a better competitor of pS7a.5-119 than was pV4a.5-138 (wild type), because the transcription of the $tRNA^{Ser}_{7}$ gene was lowered more by the 5'-flanking TNNCT mutant than pV4a.5-138 (right panel, Figure 30-B). This difference became apparent in the presence of 10 ng of competitor DNA and increased with greater amounts of competitor in the reaction. When this experiment was repeated using 10-50 ng of competitor DNA, but with 25 and 50 ng of pS7a.5-119, similar results were obtained (data not shown). These results indicated that the 5'-flanking TNNCT mutant competed better than the wild type for the S-100 transcription factor(s) even though this altered template had a lower Vmax than the wild type; this was unexpected. The data implied that complexes on the mutant template were stable.

B. Preformed complexes

To assess the relative stability of complexes formed on wild type and 5'-flanking TNNCT mutant templates, preformed complexes of pV4a.5-138,-38G,-35A and pV4a.5-138 wild type were used in competition with pS7a.5-119. Complexes were formed with 40 ng of pV4a.5-138 wild type and mutant templates and at 0, 10, 20, 30, 40 and 50 min following incubation with the S-100 extract, 10 ng of pS7a.5-119 was added to the reactions. Transcription was allowed to proceed for a further 60 min before termination and analysis of products as described in Materials and Methods (Figure 31-A). Following autoradiography, the amount of product

Figure 31-A Transcription competition between preformed complexes of pV4a.5-138-38G,-35A and pS7a.5-119

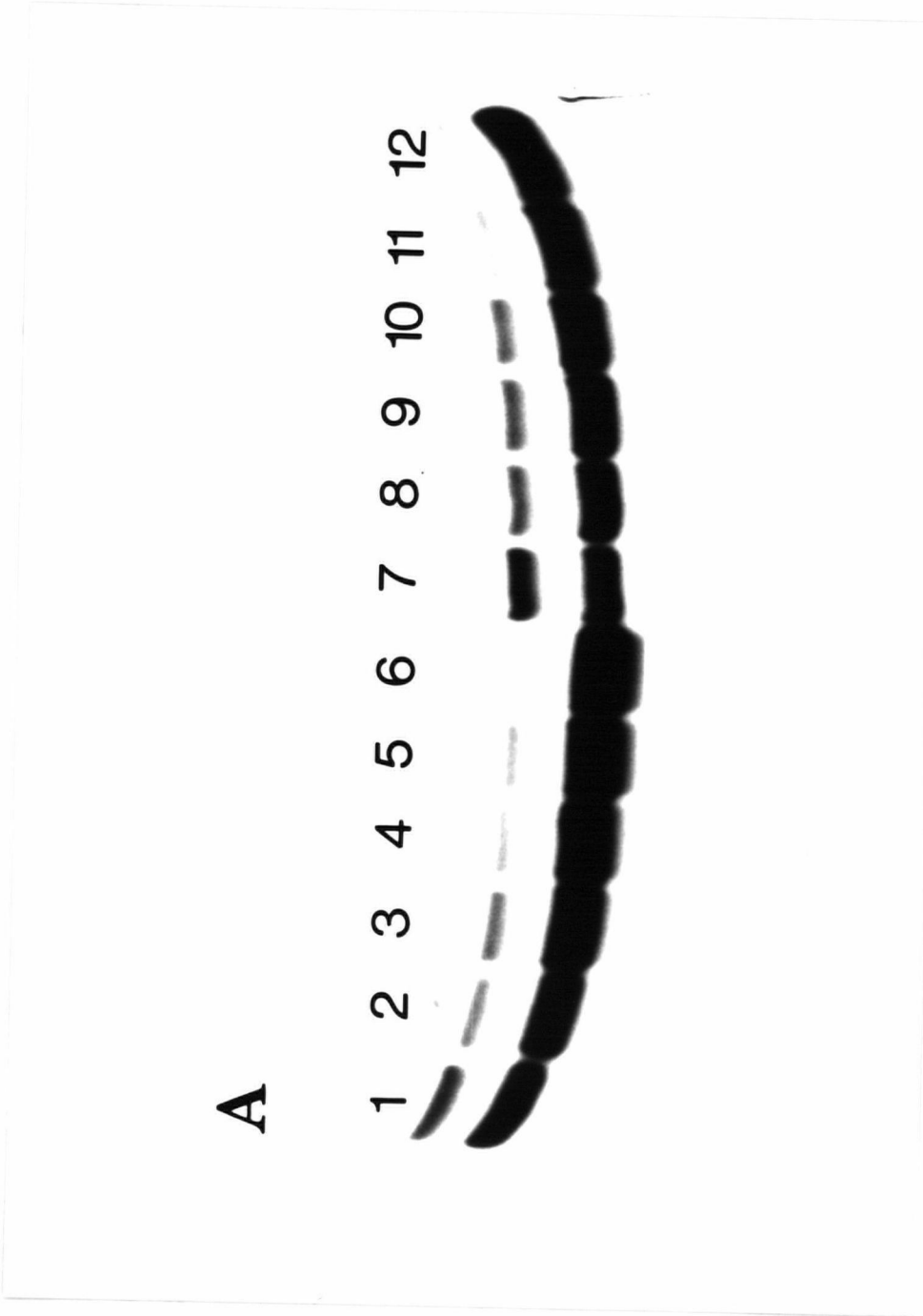
The autoradiogram shows the electrophoretic separation of labelled tRNAs from 40 ng preformed complexes of pV4a.5-138 wild type (lanes 1-6, lower band) and pV4a.5-138,-38G,-35A (lanes 7-12, lower band) in competition with 10 ng of pS7a.5-119 (lanes 1-12, upper band). All lanes contained pUC13 DNA to a total of 1.0 ug. Complexes of wild type and mutant pV4a.5-138 were formed by the addition of the S-100 extract to the template and following incubation at 23.5° C for 0, 10, 20, 30, 40 and 50 min (lanes 1-6 and 7-12 respectively), pS7a.5-119 template was added and transcriptions were allowed to continue for an additional 60 min prior to termination and analysis of products on a 10% polyacrylamide gel as described in Materials and Methods.

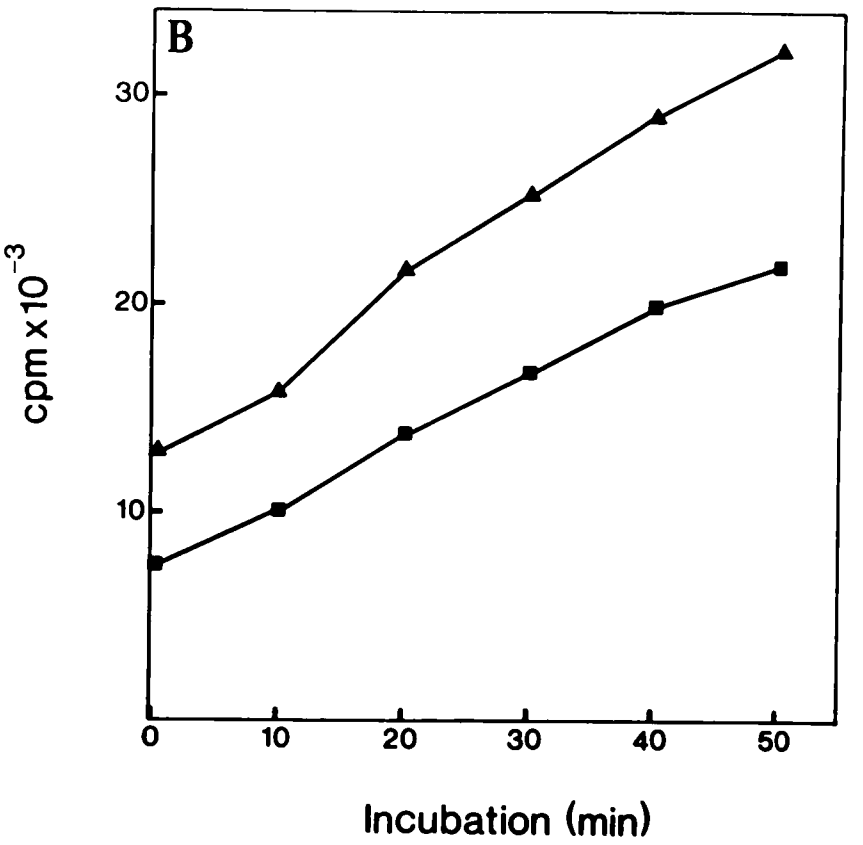
B Graph of data from transcription competition between preformed complexes of pV4a.5-138 wild type (▲), pV4a.5-138, -38G,-35A (■) and pS7a.5-119

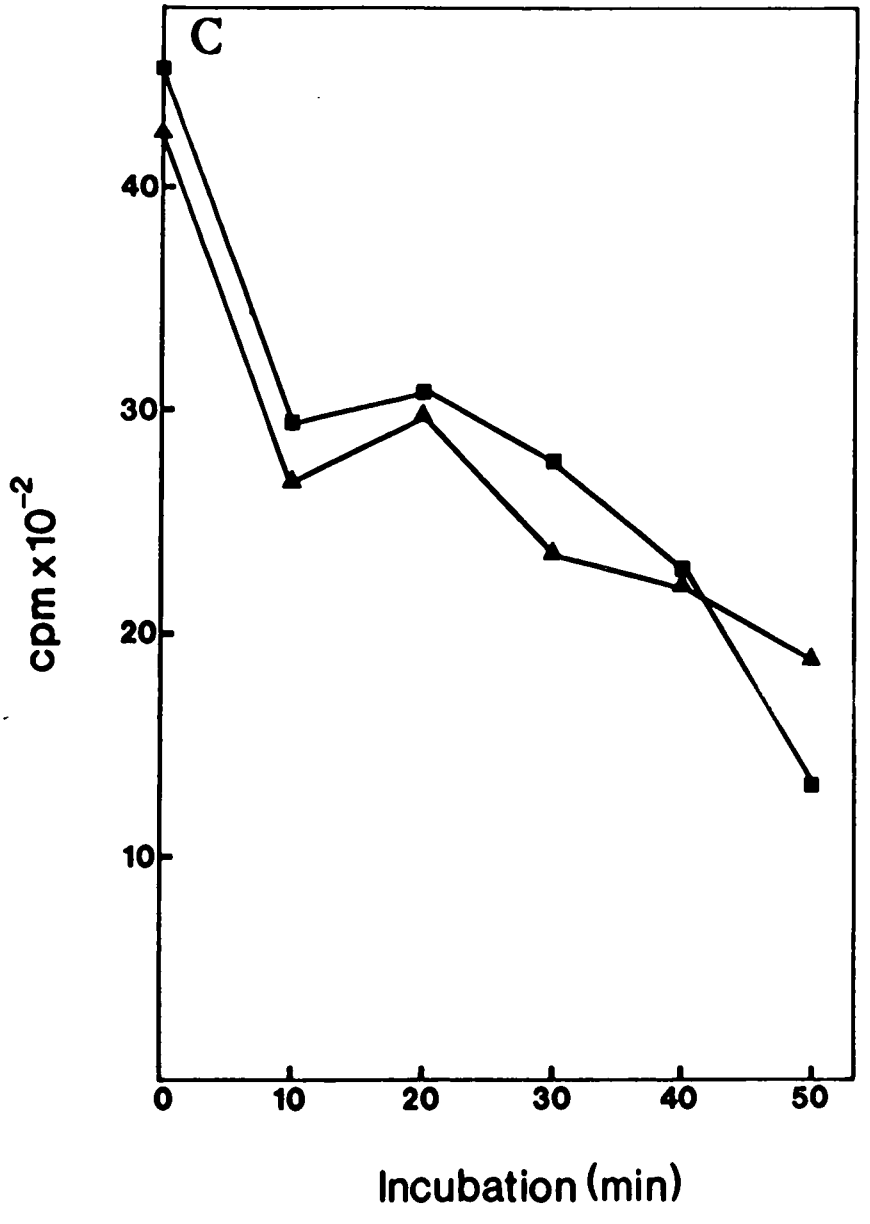
Gel slices corresponding to transcription products of pV4a.5-138 wild type and mutant templates (Figure 31-A, lanes 1-12, lower bands) were excised and the amount of product was determined by Cerenkov counting. The data were plotted as cpm of transcript per reaction versus incubation time for pV4a.5-138 wild type and mutant templates in the S-100 extract prior to the addition of pS7a.5-119 DNA.

C Graph showing the effect of competition between preformed complexes of pV4a.5-138 wild type (▲) and pV4a.5-138,-38G, -35A (■) on the transcription of pS7a.5-119

Gel slices corresponding to transcription products of pS7a.5-119 in the presence of pV4a.5-138 wild type and mutant preformed complexes (Figure 31-A, lanes 1-12, upper bands) were excised and the amount of product was determined by Cerenkov counting. The data were plotted as cpm of transcript versus incubation time for complexes of pV4a.5-138 in S-100 extract prior to the addition of pS7a.5-119.


A
1 2 3 4 5 6 7 8 9 10 11 12


B
30
20
10
cpm x10^{-3}
0
10
20
30
40
50
Incubation (min)


C
40
30
20
10
cpm x10^{-2}
0
10
20
30
40
50
Incubation (min)

produced from both $tRNA^{Val}_4$ and $tRNA^{Ser}_7$ templates was determined and the data plotted. Figure 31-B shows the data obtained from transcription of preformed complexes of pV4a.5-138 mutant and wild type templates when pS7a.5-119 DNA was added to the reactions. The graph showed that the transcription from wild type template increased faster with time than transcription from the 5'-flanking TNNCT mutant. When the ratio of slopes from the graph (Figure 31-B) was compared to the ratio of slopes for an experiment where no competitive DNA was present (see Figure 37-C; slopes were determined within the same time interval in the linear range of transcription) only a ~10% difference was observed, suggesting that neither complex was affected by the $tRNA^{Ser}_7$ gene. When the amount of $tRNA^{Ser}_7$ transcripts were quantified (Figure 31-C), the results showed that transcription of pS7a.5-119 was equally affected by both the mutant and wild type pV4a.5-138 templates as seen by a sharp decrease in $tRNA^{Ser}_7$ transcription in the presence of either wild type or mutant pV4a.5-138. This indicated that complex formation occurred effectively for both mutant and wild type Val_4 genes and that formed complexes on pV4a.5-138,-38G,-35A were equally stable. Therefore the TNNCT in the 5'-flank did not appear to affect complex stability.

VI. Effect of 140 mM NaCl on the transcription of pV4a.5-138 and pV4a.5-138,-38G,-35A

Since Stillman et al. (1984a) had previously shown that 140 mM NaCl negatively affected the binding of transcription factors to tRNA genes, NaCl was used in the following experiments to determine whether any differences might exist in the complexes formed on the wild type and 5'-flanking TNNCT mutant $tRNA^{Val}_4$ genes.

A. Effect on transcription rate

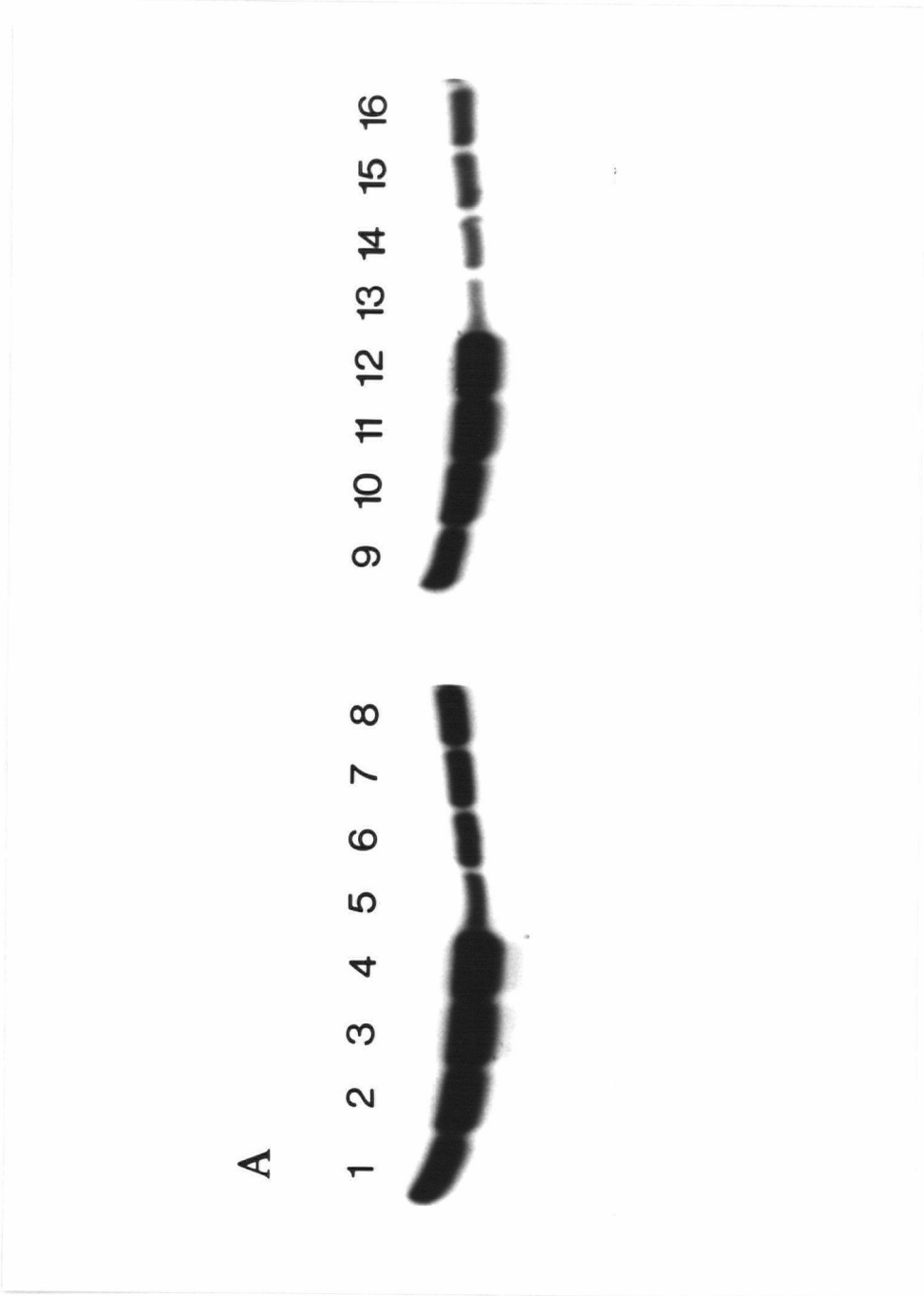
To determine the influence of 140 mM NaCl on the rate of transcription of pV4a.5-138 mutant and wild type templates, 10, 20, 33, and 50 ng of template DNA was preincubated with the S-100 extract for 30 min. Following the incubation for 30 min, NaCl was added to a final concentration of 140 mM and transcription continued for an additional 60 min. Reactions were terminated and analyzed for products as described in Materials and Methods. The control transcriptions contained an equal volume of dH_2O substituted for the volume of NaCl added to the reactions (Figure 32-A). The amount of product for each reaction was determined and the data plotted (Figure 32-B). When Vmax values for mutant and wild type templates were calculated, the results showed a 76% decrease in Vmax for pV4a.5-138 wild type and a 85% decrease in Vmax for the mutant pV4a.5-138,-38G,-35A templates. Therefore 140 mM NaCl severely decreased the rate of transcription of both wild type and

Figure 32-A Effect of 140 mM NaCl on transcription rates of pV4a.5-138 and pV4a.5-138,-38G,-35A

The autoradiograms show the electrophoretic separation of labelled tRNAs from pV4a.5-138 wild type (lanes 1-8) and pV4a.5-138,-38G,-35A mutant (lanes 8-16) templates. Lanes 1-4, 5-8, 9-12 and 13-16 represent the transcription products of 10, 20, 33 and 50 ng of template DNA which had been preincubated with S-100 extract prior to the addition of NaCl (lanes 5-8 and 13-16) or an equal volume of dH_2O (lanes 1-4 and 9-12). Transcription was continued for another 60 min before termination. All transcriptions were carried out using a total DNA concentration of 1.0 ug per reaction at 23.5° C and transcription products were analyzed on 8% polyacrylamide gels as described in Materials and Methods.

B Graph of data showing the effect of 140 mM NaCl on transcription rates of pV4a.5-138 (▲) and pV4a.5-138,-38G, -35A (■)

Gel slices corresponding to transcription products of pV4a.5-138 wild type (left panel) and mutant (right panel) templates in the presence of 140 mM NaCl (figure 32-A) were excised and the amount of product was determined by Cerenkov counting. The data were plotted as cpm of transcript per hour of reaction versus template DNA input. The presence (+) or absence (-) of NaCl is shown next to the curves.


A
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16

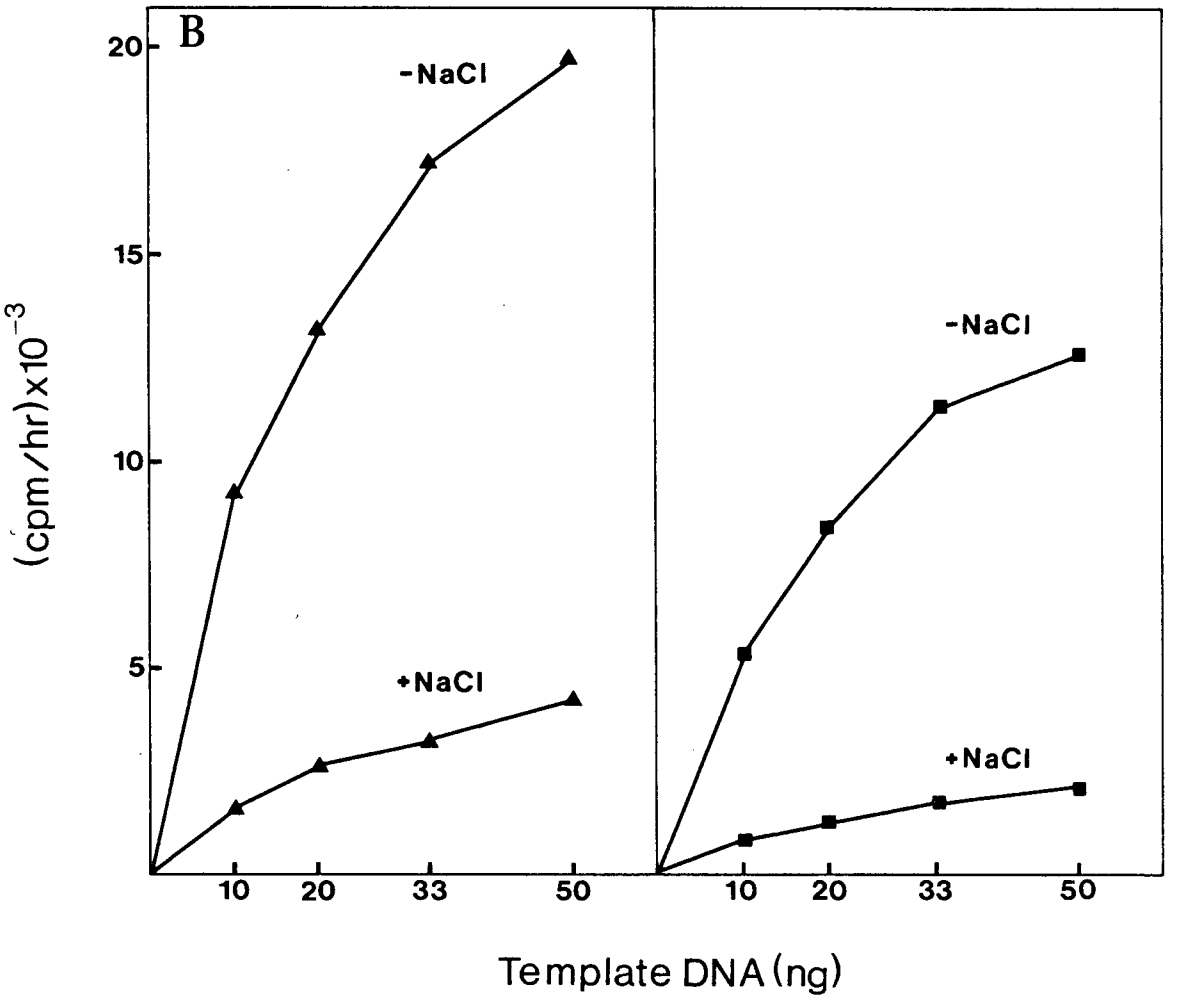

B
20
15
10
5
(cpm/hr)x10^{-3}
-NaCl
+NaCl
-NaCl
+NaCl
10
20
33
50
10
20
33
50
Template DNA (ng)

Figure 33-A Time course of the effect of NaCl during transcription of pV4a.5-138 wild type

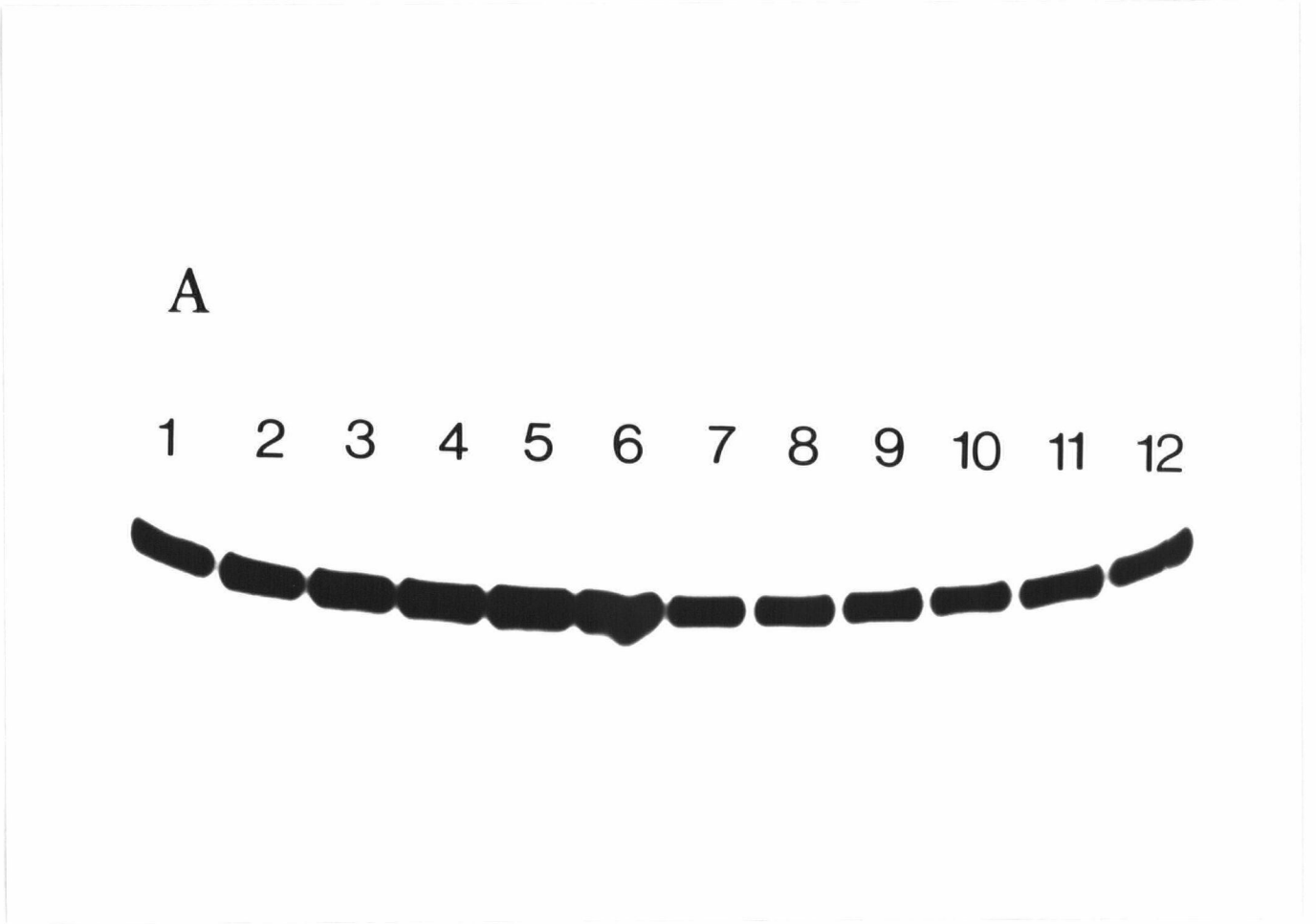
The autoradiogram shows the electrophoretic separation of labelled tRNAs from pV4a.5-138 wild type. 40 ng of template were incubated with the S-100 extract for 60 min until reactions were in the linear range of transcription and NaCl was added to the reactions (lanes 7-12) to a final concentration of 140 mM. An equal volume of dH_2O was added to a second set of reactions (lanes 1-6). Reactions were continued for 10, 20, 30, 40, 50 and 60 min (lanes 1-6 and 7-12 respectively) before termination. All transcriptions were carried out using a total DNA concentration of 1.0 ug per reaction at 23.5° C and products were analyzed on an 8% polyacrylamide gel as described in Materials and Methods.

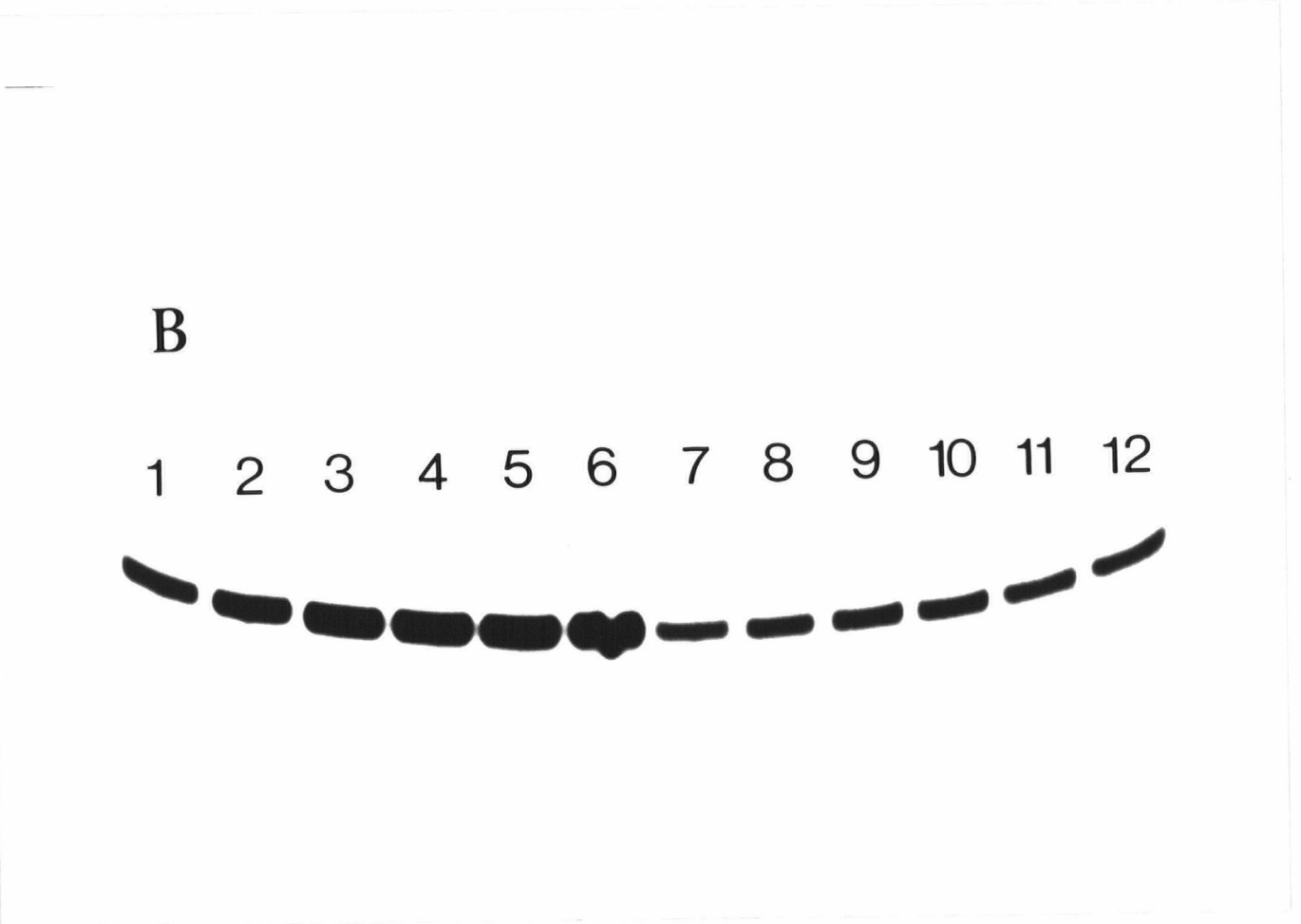
B Time course of the effect of NaCl during the transcription of pV4a.5-138,-38G,-35A

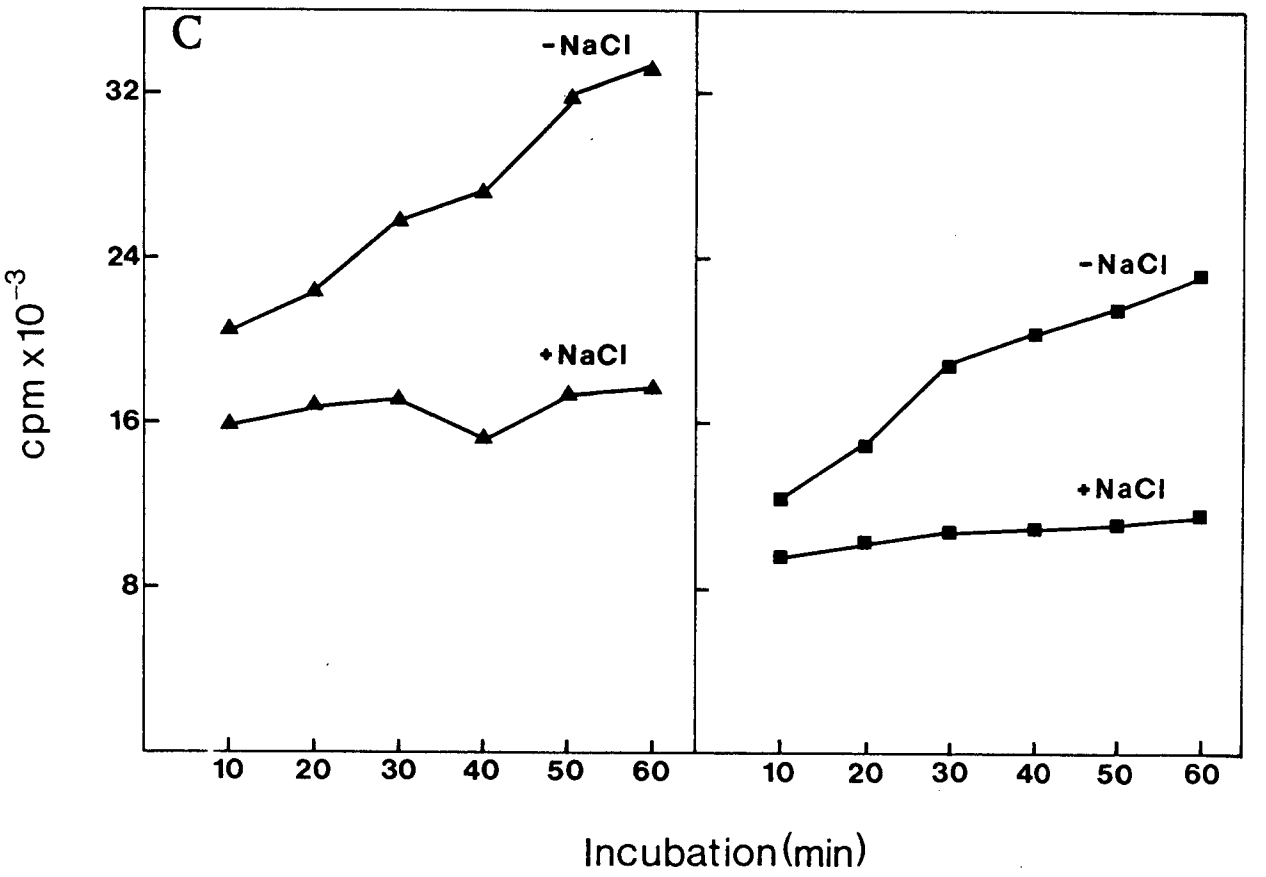
The autoradiogram shows the electrophoretic separation of labelled tRNAs from pV4a.5-138,-38G,-35A. Conditions used were identical to those described in Figure 33-A. Lanes 13-18 and 19-24 refer to transcriptions in the presence of dH_2O or 140 mM NaCl for 10, 20, 30, 40, 50 and 60 min respectively.

C Graph of data showing the effect of 140 mM NaCl on the transcriptions of pV4a.5-138 wild type (▲) and pV4a.5-138 -38G,-35A (■) over time

Gel slices corresponding to transcription products of pV4a.5-138 wild type (Figure 33-A) and mutant (Figure 33-B) templates following transcription in the presence of 0 or 140 mM NaCl were excised and the amount of product determined by Cerenkov counting. The data were plotted as cpm of transcript versus incubation time of pV4a.5-138 wild type (left panel) and pV4a.5-138 mutant (right panel) templates in the presence of 140 mM NaCl. The presence (+) or absence (-) of NaCl is shown next to the curves.


A
1 2 3 4 5 6 7 8 9 10 11 12


B
1 2 3 4 5 6 7 8 9 10 11 12


C
-NaCl
+NaCl
32
24
16
8
cpm x10^{-3}
10
20
30
40
50
60
-NaCl
+NaCl
Incubation(min)

5'-flanking mutant templates by destabilizing their transcription complexes.

B. Effect on transcription over time

A time course of the effect of 140 mM NaCl was carried out to assess any differences which may exist between the times at which complexes on wild type and 5'-flanking TNNCT mutant $tRNA^{Val}_4$ gene became susceptible to the NaCl.

To determine the time course for the effect of NaCl on transcription, complexes were formed with 40 ng of pV4a.5-138 wild type and mutant templates for 60 min and NaCl added to a final concentration of 140 mM. Transcriptions were terminated at 10, 20, 30, 40, 50 and 60 min following the addition of NaCl and the products were analyzed as described in Materials and Methods (Figure 33-A). The control transcriptions contained an equal volume of dH_2O substituted for the volume of NaCl added (Figure 33-B). The amount of product for each reaction was quantitated and the data plotted (Figure 33-C). The lower lines in both panels of the figure refer to transcription in the presence of 140 mM NaCl. It was observed that for the reactions containing no NaCl, transcription increased by approximately 50% for both pV4a.5-138 (upper line, left panel) and 5'-flanking mutant (upper line, right panel) templates over the 60 min interval. However, only a ~10% increase in transcription was observed in the presence of NaCl for both wild type and mutant templates over the 60 min time range showing that that transcription was severely affected by 140 mM NaCl in

both pV4a.5-138 wild type and pV4a.5-138,-38G,-35A mutant templates within ten minutes following the addition of NaCl. Therefore, the TNNCT mutant did not display greater sensitivity to the effects of 140 mM NaCl than pV4a.5-138.

C. NaCl concentration curve

The previous experiments (outlined above) had determined that NaCl at a concentration of 140 mM severely inhibited transcription rates and complex stabilities of both wild type and 5'-flanking mutant $tRNA^{Val}_4$ genes. To examine the effects of NaCl at different concentrations on the transcriptions of pV4a.5-138 and pV4a.5-138,-38G,-35A, 40 ng of wild type and mutant templates were incubated with the S-100 extract for 60 min at which time NaCl was added to final concentrations of 0, 30, 60, 90, 120 and 140 mM. Transcriptions were continued for a further 60 min at 23.5° C following the addition of NaCl and the products analyzed as described in Materials and Methods (Figure 34-A). The amount of product for each reaction was determined and the data plotted (Figure 34-B). The results showed that transcription decreased by approximately 51% for both pV4a.5-138 and pV4a.5-138,-38G,-35A templates over the NaCl concentration range indicating that complexes were equally affected. Therefore the complexes formed on the mutant template did not appear to be more sensitive to ionic strength than wild type complexes.

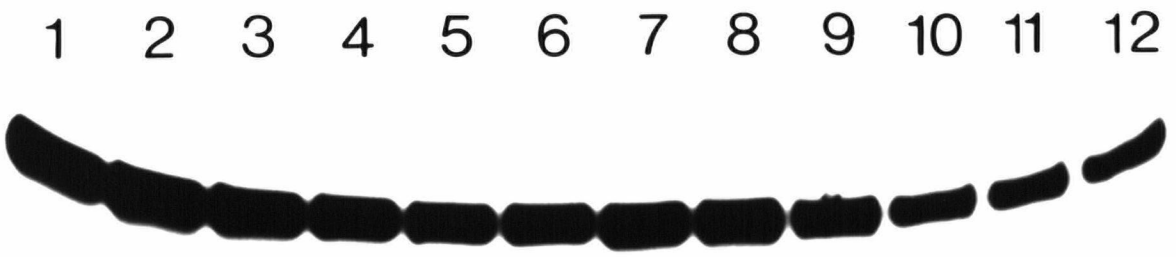
Figure 34-A Effect of different NaCl concentrations on the transcriptions of pV4a.5-138 wild type and and pV4a.5-138,-38G,-35A

The autoradiogram shows the electrophoretic separation of labelled tRNAs from pV4a.5-138 wild type (lanes 1-6) and mutant (lanes 7-12) templates. 40 ng of template DNA were incubated with the S-100 extract for 60 min until reactions were in the linear range of transcription and NaCl was added to final concentrations of 0, 30, 60, 90, 120 and 140 mM (lanes 1-6 and 7-12 respectively). Reactions were terminated after 60 min and the products separated on an 8% polyacrylamide gel as described in Materials and Methods. All transcriptions were carried out using a total DNA concentration of 1.0 ug per reaction at 23.5° C.

B Graph of data showing the effects of varying concentrations of NaCl on the transcriptions of pV4a.5-138 (▲) and pV4a.5-138,-38G,-35A (■)

Gel slices corresponding to transcription products of pV4a.5-138 wild type and mutant templates (Figure 34-A) in the presence of different NaCl concentrations were excised and the amount of product determined by Cerenkov counting. The data were plotted as cpm of transcript versus NaCl concentration.

A



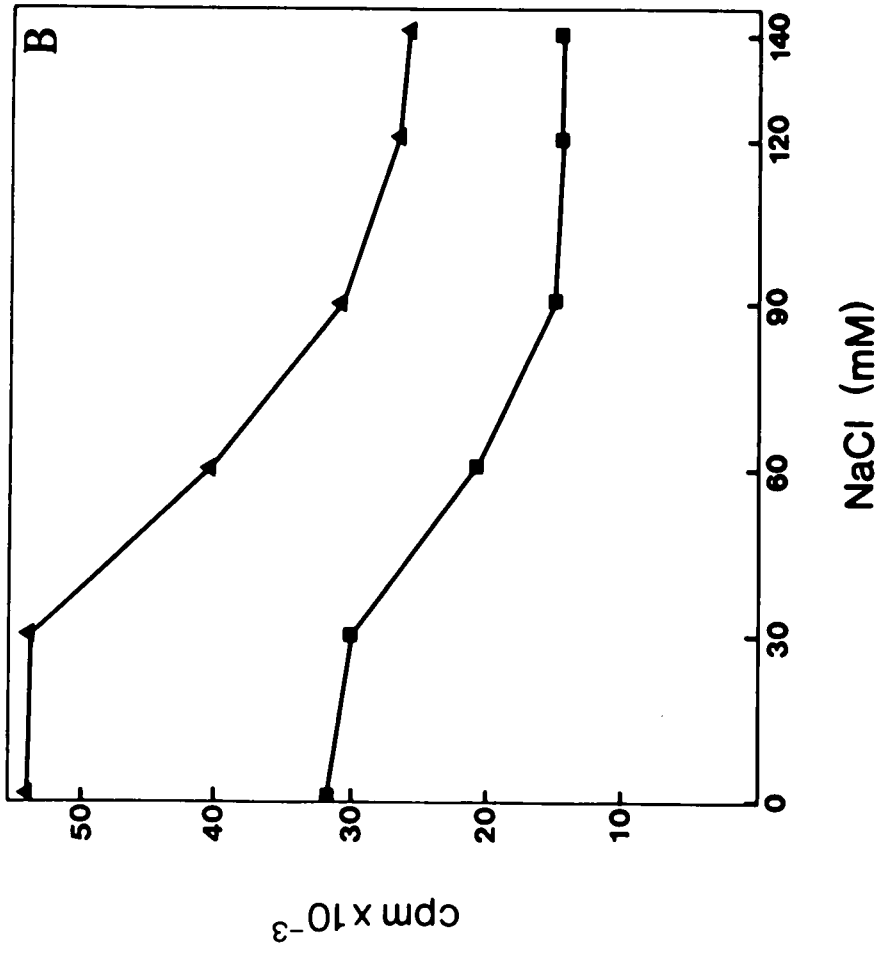
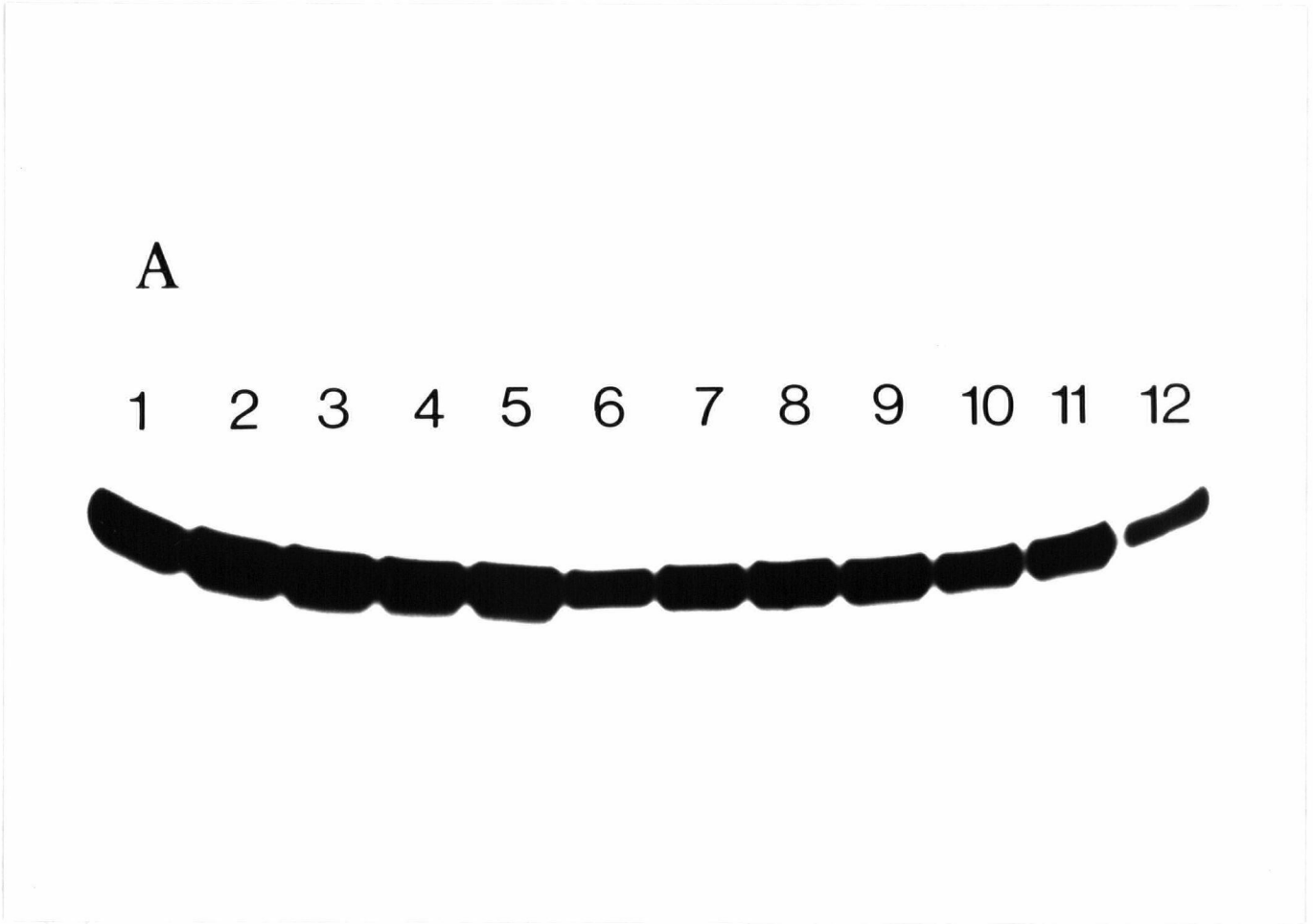

B
cpm x 10⁻³
50
40
30
20
10
0
30
60
90
120
140
NaCl (mM)

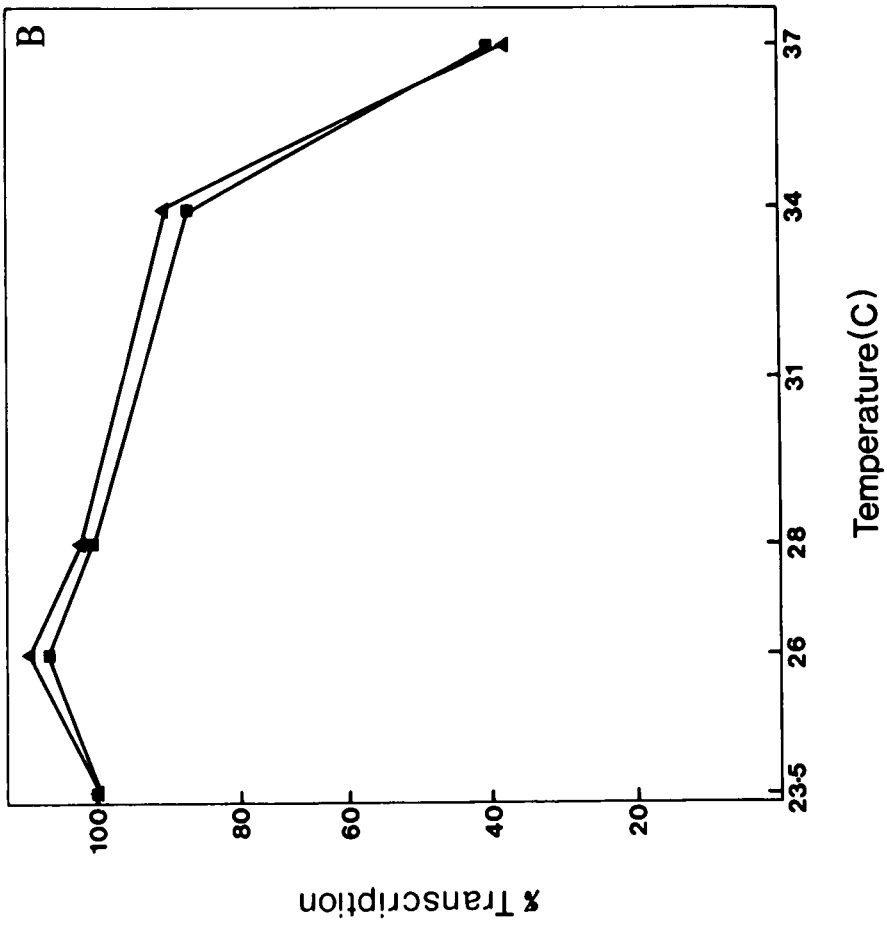
Figure 35-A Effect of increasing temperature on the transcriptions of pV4a.5-138 wild type and pV4a.5-138,-38G,-35A

The autoradiogram shows the electrophoretic separation of labelled tRNAs from pV4a.5-138 wild type (lanes 1-6) and mutant (lanes 7-12) templates. 40 ng of template DNA were incubated with the S-100 extract for 60 min at 23.5° C until reactions were in the linear range of transcription. Reactions were then transferred to incubators preset at 23.5, 26, 28, 31, 34 and 37° C (lanes 1-6 and 7-12 respectively). Reactions were terminated after 60 min and the products separated on a 8% polyacrylamide gel as described in Materials and Methods. All transcriptions were carried out using a total DNA concentration of 1.0 ug per reaction.

B Graph of data showing the effect of various temperatures on preformed complexes of pV4a.5-138 (▲) and pV4a.5-138, -38G,-35A (■)

Gel slices corresponding to transcription products of pV4a.5-138 wild type and mutant templates (Figure 35-A) at various temperatures were excised and the amount of product determined by Cerenkov counting. Data corresponding to the cpm of transcript were corrected to percent transcription relative to the amount of product at 23.5° C and plotted against temperature. Points corresponding to transcription at 31° C were omitted due to temperature fluctuations of the incubator used in this experiment.


A
1 2 3 4 5 6 7 8 9 10 11 12


B
% Transcription
100
80
60
40
20
23.5
26
28
31
34
37
Temperature(C)

VII. Effect of temperature on the transcription of pV4a.5-138 and pV4a5-138,-38G,-35A

A. Effect of increasing temperature on transcription

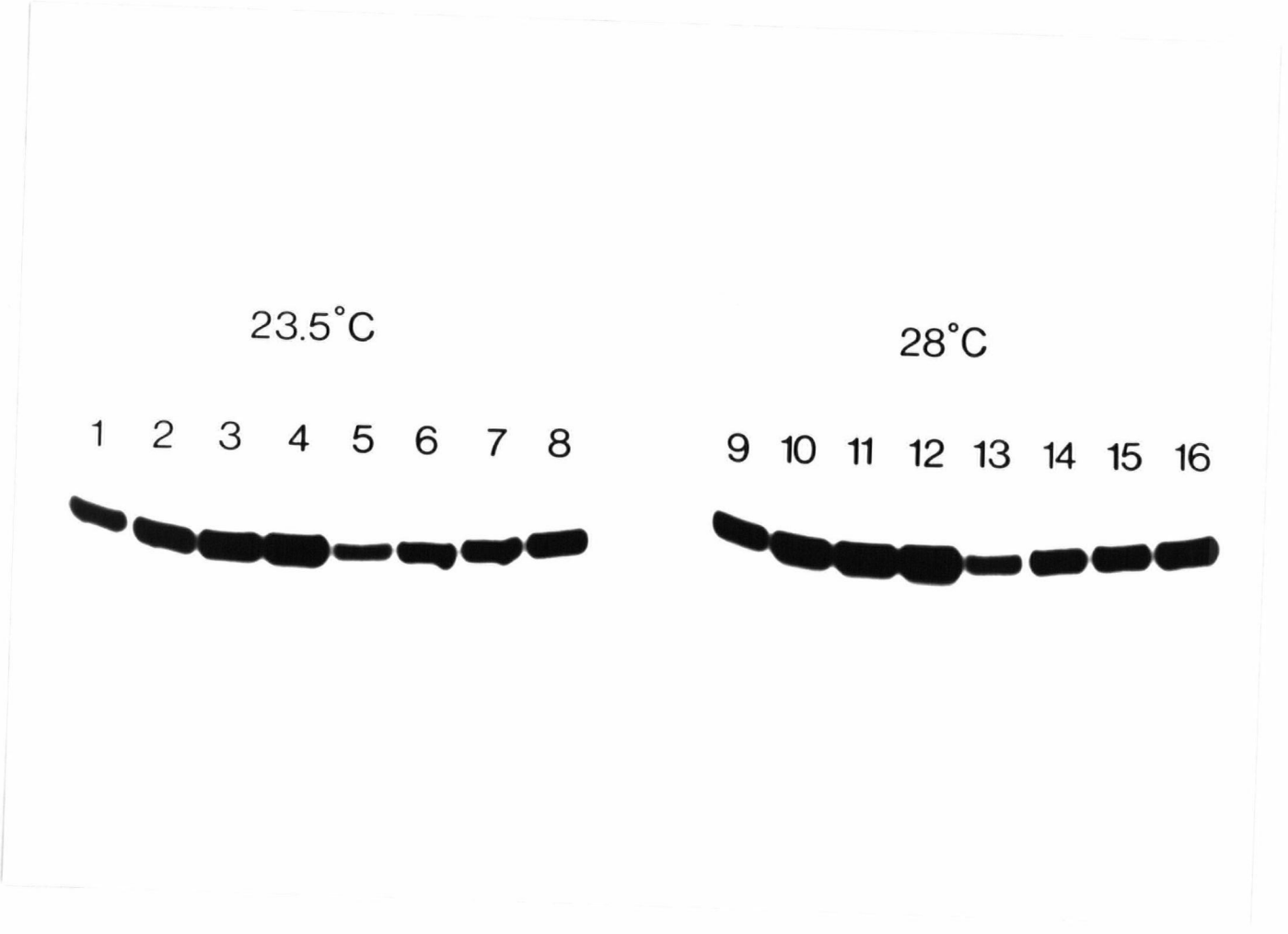
To test the effects of increased temperature on the transcription of pV4a.5-138 wild type and 5'-flanking TNNCT mutant, 40 ng of template DNA were incubated with the S-100 extract at 23.5° C for 60 min. Reactions were then incubated at 26, 28, 31, 34 and 37° C for a further 60 min before being terminated and the products analyzed as described in Materials and Methods (Figure 35-A). The percent transcription at increased temperatures relative to transcription at 23.5° C was calculated (Figure 35-B). Results showed that both the wild type and mutant were equally affected by increased temperatures. In both cases there was a slight increase at 26° C which then declined sharply at temperatures above 28° C. Therefore both the wild type and mutant complexes appeared equally susceptible to the effects of increased temperatures.

B. Effect on the rate of transcription

In this experiment complexes were formed in the presence of S-100 extract, with 10, 20, 33 and 50 ng of pV4a.5-138 wild type and mutant template DNA for 30 min at 23.5° C. The reactions were transferred to 28° C for a further 60 min and products were analyzed as described in Materials and Methods (Figure 36). Following quantification of tRNA products from reactions at 23.5 and 28° C, values corresponding to the velocity of transcription (cpm per

Figure 36 Autoradiogram of products from the transcriptions of pV4a.5-138 wild type and pV4a.5-138,-38G,-35A at 23.5 and 28° C

Autoradiograms representing the electrophoretic separation of labelled tRNAs from pV4a.5-138 wild type (lanes 1-4 and 9-12) and mutant (lanes 5-8 and 13-16) templates are shown. Transcriptions were carried out for 60 min using a total DNA concentration of 1.0 ug per reaction at 23.5° C (lanes 1-8) and 28° C (lanes 9-16) following a 30 min incubation at 23.5° C. Transcription products were analyzed on 8% polyacrylamide gels as described in Materials and Methods. Lanes 1-4, 5-8, 9-12 and 13-16 show the transcription products of 10, 20, 33 and 50 ng of template DNA respectively.


23.5°C
28°C
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16

hour) were used to derive Vmax values for the wild type and mutant templates. The increase in temperature resulted in a 26.1% increase in Vmax for pV4a.5-138 and a 12.4% increase in Vmax for pV4a.5-138,-38G,-35A relative to transcription at 23.5° C. Although the Vmax increased for both templates, the relative increase in transcription was less for the 5'-flanking TNNCT mutant than the wild type by two fold.

VIII. Analysis of the time course for the transcription of pV4a.5-138 wild type and pV4a.5-138,-38G,-35A

To examine the transcription of pV4a.5-138 wild type and 5'-flanking TNNCT mutant during the course of a 2 hr reaction, 40 ng of template DNA were incubated with the S-100 extract in a standard reaction containing a total of 1.0 ug of DNA at 23.5° C as described in Materials and Methods. At 10 min intervals reactions were terminated and the products electrophoresed on polyacrylamide gels (Figure 37-A and B). Following autoradiography, the amount of product for each reaction was determined and values corresponding to the velocity of transcription were plotted against reaction times (Figure 37-C). Results showed that transcription was detectable within 10 min of the addition of S-100 extract, in both the wild type and mutant templates. In addition, both templates showed a non-linear curve for transcription. Extrapolation from the line representing the linear portion of the curves to the axis showed the intercepts to be at

Figure 37-A Time course of transcription for pV4a.5-138

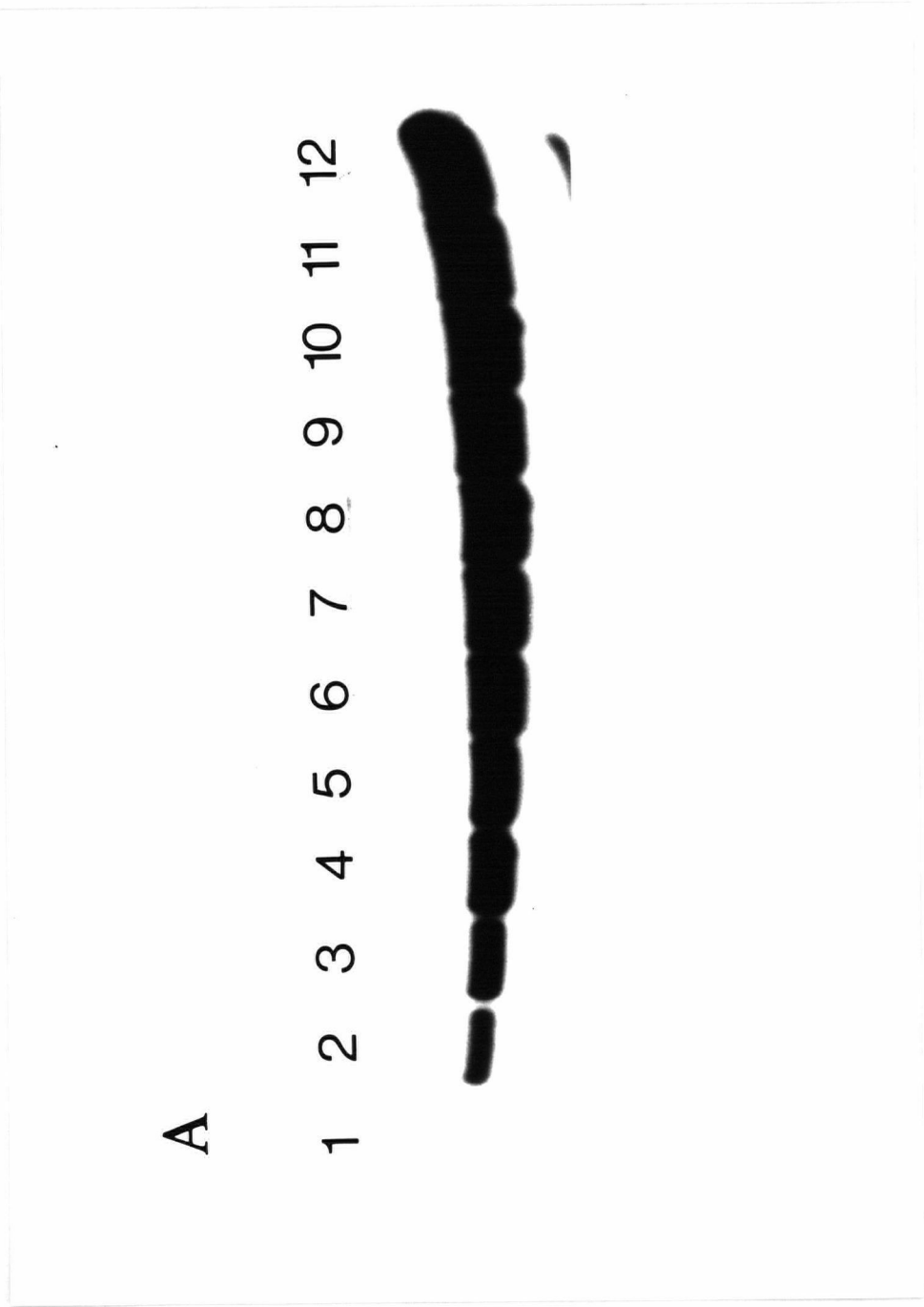
The autoradiogram shows the electrophoretic separation of labelled tRNAs from pV4a.5-138 wild type. Transcriptions were carried out with 40 ng of template DNA for 2 hrs at 23.5° C. Reactions were terminated at 10 min intervals and electrophoresed on an 8% denaturing polyacrylamide gel as described in Materials and Methods. Lanes 1-12 represent transcription products from 10, 20, 30, 40, 50, 60, 70, 80 90, 100, 110 and 120 min of reaction respectively.

B Time course of transcription for pV4a.5-138,-38G,-35A

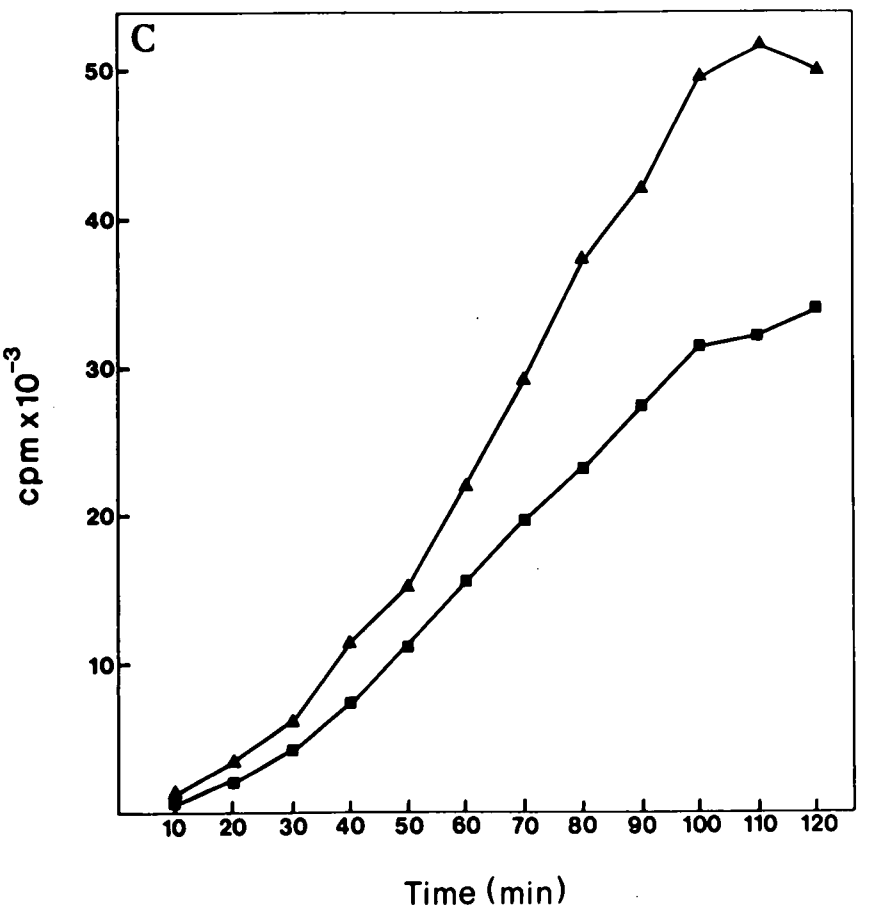
The autoradiogram shows the electrophoretic separation of labelled tRNAs fro pV4a.5-138 mutant in 5'-flanking TNNCT. Conditions for transcription and analysis of products were as described in Figure 37-A.

C Graph showing the time course of transcription for pV4a. 5-138 wild type (▲) and pV4a.5-138,-38G,-35A (■)

Gel slices corresponding to transcription products of pV4a.5-138 wild type and mutant templates (Figure 37-A and B) at various time points during the course of transcription were excised and the amount of product determined by Cerenkov counting. Data was plotted as cpm of transcript versus reaction time.


A
1 2 3 4 5 6 7 8 9 10 11 12


B
1 2 3 4 5 6 7 8 9 10 11 12


C
50
40
30
20
10
cpm x10^{-3}
10 20 30 40 50 60 70 80 90 100 110 120
Time (min)

approximately the same position. Therefore no significant lag in initiation time of the 5'-flanking TNNCT mutant relative to the wild type template was observed. The slope of the transcription curve for the mutant was lower than the slope of that for the wild type. Since it was possible that the rate of transcription initiation may have been affected, an experiment was carried out to measure specifically the rate of transcription initiation.

IX. Transcription initiation in pV4a.5-138 wild type and pV4a.5-138,-38G,-35A

It was previously shown that initiation of transcription in pV4a.5-179 occurred at position -9G (Sajjadi et al., 1987). In order to analyze specifically transcription of $tRNA^{Val}_4$ gene wild type and 5'-flanking mutant templates initiating at -9G, [γ-^{32}P] GTP was substituted for [α-^{32}P] UTP in standard transcription reactions. First, a GTP concentration curve was carried out to determine the optimal concentration of GTP required in the reaction for efficient transcription. In this experiment, 40 ng of pV4a.5-138 DNA were used as template in twelve standard 90 min transcription assays (as described in Materials and Methods) containing 0 to 1000 uM GTP. Following the analysis of transcription products, the results showed that the S-100 extract contained a sufficient concentration of GTP and no additional GTP was required for efficient transcription (data not shown).

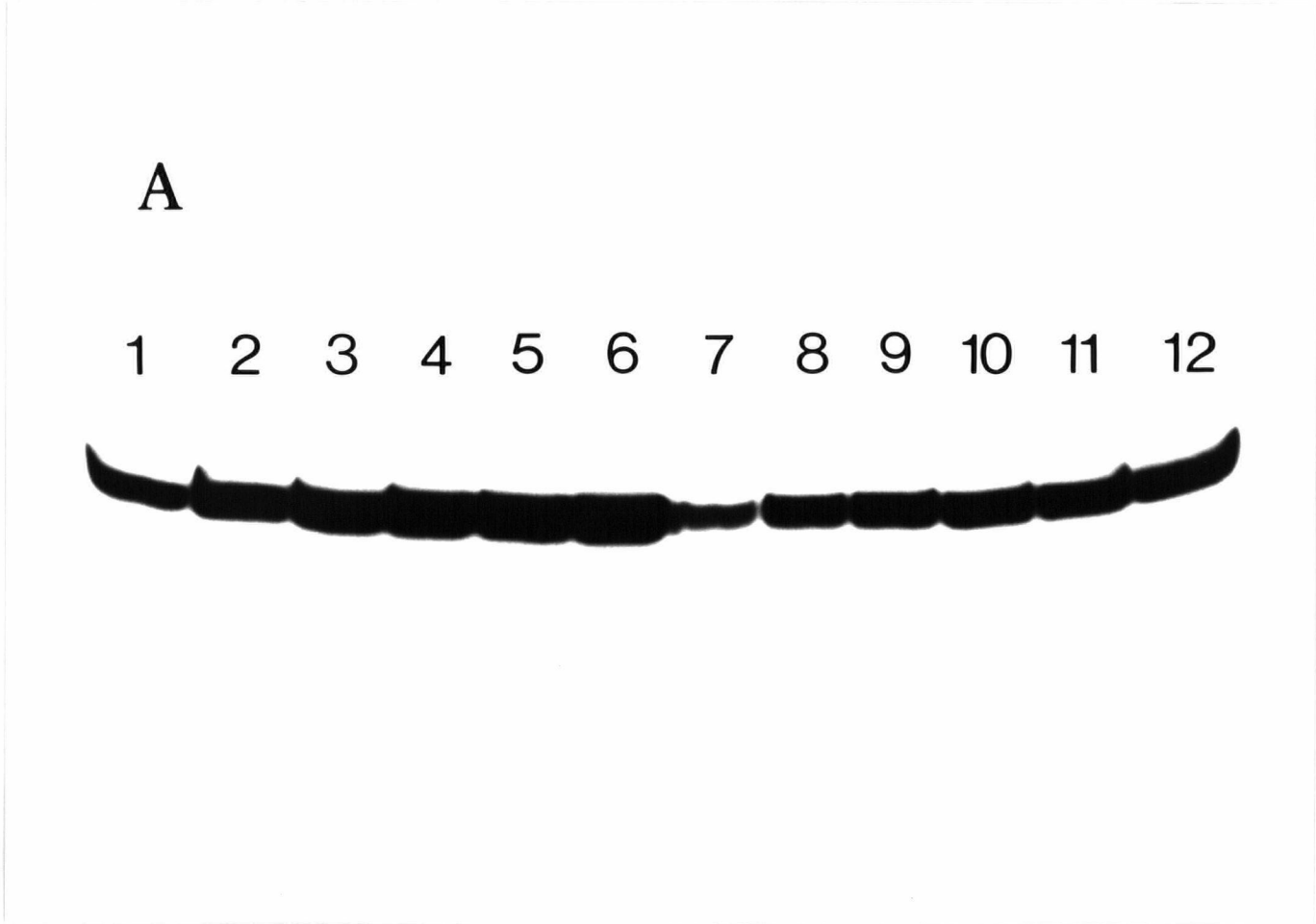
Next, transcriptions were carried out with both pV4a.5-138 wild type and 5'-flanking mutant templates at six different concentrations of input DNA as described in Materials and Methods, except that 5 uCi of [γ-^{32}P] GTP (752 Ci/mmol) was substituted for [α-^{32}P] UTP and the reactions contained 6 mM unlabelled UTP. A replicate of the experiment under standard conditions with [α-^{32}P] UTP, was carried out in parallel and served as control. Following termination of transcription, the products of the reactions were separated by electrophoresis on 15% denaturing polyacrylamide gels to detect partial transcripts (Figure 38-A and B). The amount of product for each reaction in the presence of [α-^{32}P] UTP and [γ-^{32}P] GTP was determined and the data used to derive Vmax values for each template. Although only transcripts longer than ten nucleotides could be resolved under these conditions, there was no evidence of shorter partial transcripts from the mutant template. The results showed that pV4a.5-138,-38G,-35A was defective in initiation with a 34% decrease in Vmax for reactions with [γ-^{32}P] GTP and a 39% decrease in Vmax with [α-^{32}P] UTP as the labelled nucleotide.

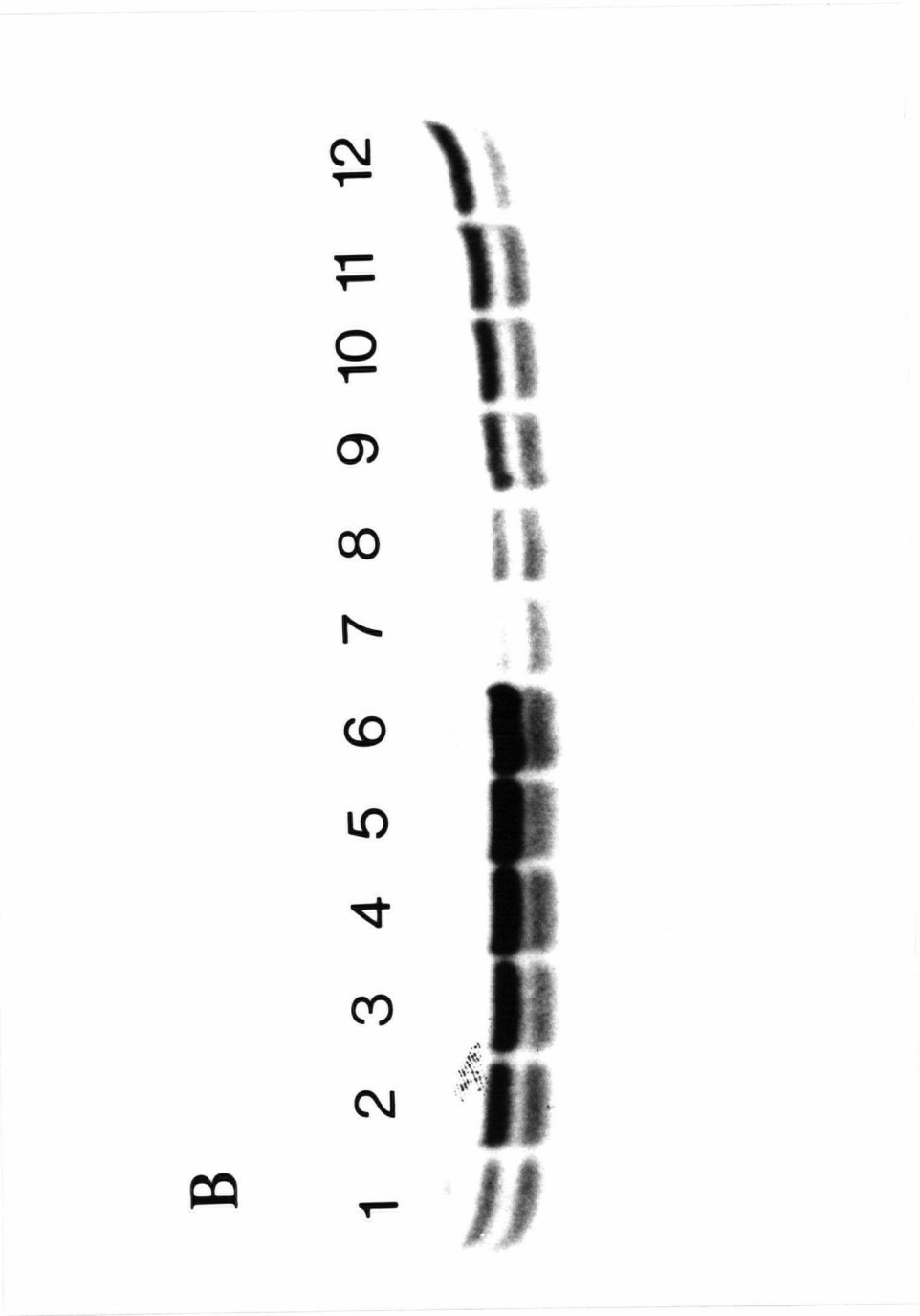
Figure 38-A Autoradiogram of products from transcriptions of pV4a.5-138 and pV4a.5-138-38G-35A with [α-^{32}P] UTP

The autoradiogram shows the electrophoretic separation of labelled tRNAs from pV4a.5-138 wild type (lanes 1-6) and 5'-flanking mutant (lanes 7-12) templates. Transcriptions were carried out using a total DNA concentration of 1.0 ug per reaction at 23.5° C with labelled [α-^{32}P] UTP and transcription products were analyzed on a 15% denaturing polyacrylamide gel as described in Materials and Methods. Lanes 1-6 and 7-12 represent the transcription products of 10, 20, 33, 50, 66.7 and 100 ng of template DNA respectively.

B Autoradiogram of products from the transcriptions of pV4a.5-138 and pV4a.5-138,-38G,-35A with [γ-^{32}P] GTP

The autoradiogram shows the electrophoretic separation of labelled tRNAs from pV4a.5-138 wild type (lanes 1-6) and 5'-flanking mutant (lanes 7-12) templates. Transcription conditions were as described for Figure 38-A, except that [γ-^{32}P] GTP was used as the labelled nucleotide. The faint bands present in all lanes which appear above and below the Val_4 transcripts are not template specific (i.e. do not vary with template DNA input) and are normally well separated from the primary transcript in 8% polyacrylamide gels.


A
1 2 3 4 5 6 7 8 9 10 11 12


B
1 2 3 4 5 6 7 8 9 10 11 12

DISCUSSION

I. Mutations in 5'-flanking TNNCT

The purpose of this study was to demonstrate specifically the role of the sequence TCGCT in the modulation of pV4a.5-138 transcription and to shed light on the mechanism of its action. An extensive deletion series into the 5'-flank of pV4a.5-179 had shown that deletion of sequences to position -38 relative to the mature coding sequence of $tRNA^{Val}_4$ gene did not result in a lower Vmax than for the wild type gene (Sajjadi et al., 1987). However, deletion of one additional nucleotide resulted in a 43% decrease in Vmax and deletion of the remaining TCGCT sequence resulted in a 85% decrease in Vmax relative to pV4a.5-179. In addition, it was also demonstrated that the sequence TCGCT at position -19 did not positively modulate the level of transcription (Sajjadi, 1985). A homology search in the 5'-flank of other Drosophila tRNA genes which directed transcription efficiently did not reveal a sequence TCGCT. However, a sequence of the general form TNNCT was found and it was suggested that the pentanucleotide may be a positive modulator of tRNA gene transcription. Since some of the effects observed for the deletion end-points may have been contributed by the closer positioning of vector sequences to the gene, further studies on the effects of TCGCT in the context of a wild type flank were required. To assess precisely the contribution of conserved nucleotides

in the sequence TNNCT to efficient transcription, a number of point mutants were created in the 5'-flank of the $tRNA^{Val}_4$ gene.

Nucleotide changes were created in pV4a.5-138 which was identical to pV4a.5-179 in transcription efficiency. Both pV4a.5-138 and -45 were sub-cloned into the EcoRI/HindIII site of pEMBL8-, sequenced in their entirety and their relative transcription efficiencies determined.

Table 1 shows the list of point mutants created in pV4a.5-138. The -38G mutation resulted in a 28% decrease in Vmax relative to the wild type template. Mutations at the other conserved nucleotides resulted in approximately 32% decrease in the level of $tRNA^{Val}_4$ transcription. In addition, double mutants at the conserved nucleotides, -38G,-35G and -38G,-35A resulted in approximately 40% decrease in Vmax relative to pV4a.5-138. In contrast, the -36T mutation reduced transcription efficiency by only 1% and a mutation at the second variable position, -37A resulted in a 12% increase in Vmax relative to wild type activity. Since the C to A transversion resulted in a 12% increase in Vmax and an identical transversion at the conserved nucleotide at position -35 resulted in a 32% decrease in Vmax, the drop in transcription for the conserved nucleotide was most likely not due to the nature of the nucleotide change. The data showed that mutations in the conserved nucleotides resulted in a decrease in transcription, whereas nucleotide changes at the variable positions of TCGCT did

not significantly reduce template activity. This was the first indication that the the two nucleotides at positions -37 and -36 were indeed variable. These results are consistent with having a general sequence of the form TNNCT for a positive modulator of transcription in the 5'-flank.

It is interesting that double nucleotide changes at the conserved positions did not display transcriptional activities very much greater than some of the single point mutants (Table 1). While it may be possible that TNNCT functions as a unit, no conclusions for the absence of additive effects can be drawn at this time. A nucleotide change in TNNCT was also created in pV4a.5-45. The transcription efficiency of pV4a.5-45 had been shown to be 36% higher than that of pV4a.5-179. This was postulated to be due to the removal of upstream negative modulatory sequences (Sajjadi et al., 1987). The single transversion at position -38 resulted in only a 10% decrease in Vmax relative to pV4a.5-45 (Table 1). Note that the -38 transversion in the pV4a.5-138 template resulted in a decrease in transcription that was approximately three-fold greater than the drop observed for the pV4a.5-45 template. A possible explanation for the different effects of the -38 mutation in TNNCT of pV4a.5-45 and -138 templates may be that the inhibitory sequence previously identified in the 5'-flank of pV4a.5-179 (Sajjadi et al., 1987) primarily affected the positive modulatory role of sequences at position -45. Since this terminator-like sequence was

deleted in pV4a.5-45, the inhibitory effects on the -45 sequences were removed and the template directed efficient transcription with a reduced dependence on the function of TNNCT. The mechanism underlying the increased activity of the pV4a.5-45 template will require further study.

Table 4 contains a list of tRNA genes from Drosophila whose in vitro transcription properties have been determined. The list shows that 5'-flanking sequences of genes which do not contain a TNNCT either fail to direct transcription or transcribe at very low efficiencies in vitro. The exception, pDt5-Ser_7 will be discussed in section V. Genes containing the sequence TNNCT in their 5'-flank generally direct transcription very efficiently. While the pLeu gene appears to be an exception, the 5'-flank of this gene contains multiple T tracts which have previously been shown to be inhibitory to transcription (DeFranco et al., 1981; Dingermann et al., 1982; Sajjadi et al., 1987). In addition, in order to have an effect, it appears that the positive modulatory sequence TNNCT must be located 30 bp or further upstream from the mature coding sequence (Sajjadi, 1985). This hypothesis is supported by the observation that p48His (Cooley et al., 1984) which contains a TNNCT at position -18 in its 5'-flank, is an inefficient template for transcription. In contrast, pHis which has the TNNCT located at position -42 directs transcription efficiently. Therefore the occurrence of TNNCT in the 5'-flank would appear to be correlated with

Table 4 Correlation of transcription efficiency and the pentanucleotide TNNCT

Template	Position of TNNCT[a]	Transcription[b]	Ref.
pYH48-Arg	-38_{TTTCT}	+	1
pE1.8-Arg	$-52_{TTGCT}-40_{TATCT}$[c]	+[d]	
pDt67R-Arg	$-88_{TCTCT}-84_{TTTCT}-69_{TTGCT}-36_{TGCCT}$[c]	+[d]	
pDt17R-Arg	$-151_{TAACT}-126_{TAGCT}-106_{TGTCT}-89_{TGACT}-80_{TTCCT}-53_{TCCCT}-38_{TTGCT}-33_{TTTCT}$[c]	+[c]	
p11F-Arg	No TNNCT (to -40)	-(+)	2
p35D-Arg	No TNNCT	-(+)	2
p17D-Arg	No TNNCT	-	2
pAva4-Arg	No TNNCT[c]	-[d]	
pAsn8-Asn	$-42_{TGGCT}-37_{TGGCT}-33$	+	3
pAsn6-Asn	No TNNCT (to -48)	+(-)	3
pAsn7-Asn	No TNNCT (to -48)	-(+)	3
pHis-His	-46_{TTGCT}	+	4
p48His-His	-22_{TTGCT}	-(+)	4
pLeu-Leu	$-58_{TTGCT}-42_{TTTCT}$ (to-140)	-(+)	5
pDt39R-Lys_5	-36_{TACCT}	+	6
pDt59R-Lys_5	-38_{TTCGT}	+	6
pDt5-Ser_7	No TNNCT	+	7
pDt16H#1-Ser_{774}	-39_{TAGCT}	+(-)	7
pDt16H#5-Ser_{777}	No TNNCT	+(-)	7
pDt55-0.6-Val_4	-39_{TGGCT}	+	8
pV4a.5-179-Val_4	$-38_{TCGCT}-23_{TCTCT}$	+	9
pDt92R-Val_4	$-79_{TTACT}-48_{TTGCT}-44_{TAACT}-39_{TATCT}$	+(-)[d]	10
pDt78R-Val_{3b}	No TNNCT	-	11

Footnotes to Table 4:

[a]Numbers indicate the position of the 5'T in the TNNCT sequence in the 5'-flanking DNA, relative to the mature coding sequence (+1).

[b]Symbols for transcription efficiencies are: +=efficient (within 10% of pV4a.5-179); +(-)=inefficient (less than 15% of pV4a.5-179); -(+)=very inefficient (less than 1% of pV4a.5-179); -=no detectable transcription. For entries where evaluation of transcription efficiency is taken from the literature, the levels do not relate to pV4a.5-179.

[c]C. Newton, J.L. Leung and G.M. Tener, personal communication.

[d]L. Duncan and G.B. Spiegelman, unpublished results.

1 Schaack et al., 1984
2 Dingermann et al., 1982
3 Lofquist and Sharp, 1986
4 Cooley et al., 1984
5 Glew et al., 1986
6 De Franco et al., 1982
7 St. Louis and Spiegelman, 1985
8 Rajput et al., 1982
9 Sajjadi et al., 1987
10 Addison et al., 1982
11 Leung et al., 1984

transcription efficiency. As can be seen from Table 4, of the twenty three genes listed, fourteen have a TNNCT between positions -29 and -46. Of the genes which are transcribed at moderate to high efficiency, only three of the fifteen do not contain a TNNCT in the -29 to -46 region of the 5'-flank. Thus the association of the sequence TNNCT with genes which are active templates does not appear random, although the correlation is not perfect.

II. Factor interaction with the 5'-flank

The above experiments demonstrated that TNNCT was required for the maximum level of transcription in pV4a.5-138. Although TNNCT is only five nucleotides in length, it may still be able to sequester a transcription factor required for transcription, as shown for other eukaryotic sequences of five nucleotides in length (Hatamochi et al., 1986; Miksicek et al., 1987). To explore the possibility of transcription factor interaction with TNNCT or other sequences contained in the 5'-flank, the 5'-flank of pV4a.5-179 was isolated and subcloned into the HindIII site of pEMBL8- as previously described. The isolated 5'-flank was subsequently used in a gel retardation assay in the presence of S-100 extract (Figure 28). Results showed that while the 5'-flank was retarded in the assay in the presence of poly dI-dC, no factor binding was observed when pUC DNA was used as competitor for non-specific binding. Incubation of labelled probe with both pUC and poly dI-dC DNA failed to

produce a retarded band, suggesting that pUC DNA was able to bind and compete for bound protein on the 5'-flank, suggesting that the interaction was non-specific.

The clone containing the isolated 5'-flank was also used in a transcription competition experiment with pV4a.5-138, the wild type $tRNA^{Val}_4$ gene. Competition experiments were carried out by the addition of competitor DNA (5'-flank) both simultaneously as well as to preformed complexes of pV4a.5-138. Results showed that the 5'-flank did not reduce the transcription of the intact $tRNA^{Val}_4$ gene. The transcription competition data and the results from the gel retardation assay together suggest that the 5'-flanking sequences of pV4a.5-179 cannot bind factors required for efficient tRNA transcription when separated from the internal control regions of the gene. The results do not rule out the possibility for interaction of RNA polymerase III or other proteins with the 5'-flank, once a stable complex has been formed. Since such an interaction would require the recognition of a stable complex on the template, RNA polymerase III which has been shown to recognize these complexes prior to transcription initiation, appears to be a good candidate for interaction with TNNCT.

III. Mutations in the internal TNNCT

The $tRNA^{Val}_4$ gene contains the sequence TGCCT in the anticodon stem/loop region (Figure 13). Since the TNNCT in the 5'-flanking region of the gene was shown to be involved

in modulation of transcription, the internal TNNCT sequence was mutagenized to determine whether it too contributed to transcription efficiency. In addition, the experiment sought to explore the possibility for the requirement of TNNCT sequences in both extragenic and intragenic regions of the gene, which would shed light on the problem of 5'-flank/gene compatibility discussed earlier (section IV. of Introduction).

Three nucleotide changes were created between positions +29 and +33 of pV4a.5-138 by site-specific mutagenesis to produce a HhaI site. The newly created site would simplify the mutant screening procedure and would allow the construction of maxigenes for future studies. When full-length transcripts were quantified, the template mutant in the internal TNNCT had a Vmax 46% lower than its counterpart, pV4a.5-138 wild type. In addition, the autoradiogram showed an abundance of shorter bands present on the gel which appeared to be partial transcription products (Figure 22). It should be noted that all S-100 extracts used in this study were tested for high efficiency of transcription and the absence of processing activity under the conditions employed. Therefore if partial transcripts were also quantified along with full-length transcripts, then it is possible that no decrease in Vmax would be detected for the template mutant in the internal TNNCT, indicating no differences in the rates of initiation of wild type and mutant templates. Mutants created in the

stem/loop region of other tRNA genes have not yielded consistent results in terms of their effects on transcription rates (Sharp et al., 1985). It is conceivable that the changes reported here may have in fact increased the rates of transcription. However, this is the only report in which mutations in the anticodon stem/loop region have affected the elongation of transcripts, suggesting that the mutations may have altered the interaction of transcription factors or RNA polymerase III with the template.

The nucleotide changes in the internal TNNCT were also created in the template mutant in 5'-flanking TNNCT (pV4a.5-138,-38G,-35A) whose transcription efficiency was 42% lower than wild type. The new construct which was mutant in both 5'-flanking and internal TNNCT sequences had a transcription efficiency 54% lower than pV4a.5-138 wild type when full-length transcripts were quantified. Faint bands corresponding to shorter bands from the internal TNNCT mutant were also present in the gel from the template mutant in both TNNCTs (Figure 23).

The decrease in the level of full-length transcription for 5'-flanking and internal TNNCT sequences was not additive. The drop in Vmax may represent, for the greater part, the level of transcription for a template lacking TNNCTs in the 5'-flank alone.

It is unlikely that mutating the internal TNNCT is responsible for specifically affecting the site of

initiation of transcription, since the size of the shorter bands would require initiation to occur within the internal control sequences containing bound transcription factors. A possible explanation for the premature termination of transcripts is that the internal TNNCT contributes to the interaction of factors with the template and that the shorter bands represent partial transcripts resulting from unstable complexes. Stewart et al. (1985) created two nucleotide changes in the anticodon of a Drosophila $tRNA^{Arg}$ gene between positions +34 and +36 which resulted in a 25% decrease in the level of transcription. However, complex formation for this gene as measured in a competition assay with a reference competitor was reported to be at almost wild type levels. The $tRNA^{Arg}$ gene also contained a TNNCT between position +29 and +33, which was not mutagenized. While these results show that the anticodon stem/loop region is important in transcription, the mechanism is open to speculation at this time.

IV. Transcription properties of a template mutant in 5'-flanking TNNCT

The experiments outlined above showed that the sequence TNNCT contained in the 5'-flank of pV4a.5-138 was required for efficient tRNA transcription. However, it was not known whether TNNCT function was related to its role in influencing complex formation or in directly affecting the efficiency of transcription initiation. The present model

for the formation of complexes on tRNA genes is based on data obtained from the transcription of Xenopus 5S RNA genes (Lassar et al., 1983; Setzer and Brown, 1985). Studies have shown that complex formation is initiated by the binding of TFIIIC to the T control region (Johnson-Burke and Soll, 1985), which occurs within one minute following the addition of extract to the template. The interaction results in a metastable complex which is stabilized by the binding of TFIIIB (Lassar et al., 1983; Johnson-Burke and Soll, 1985). The result is a stable "preinitiation" complex which is in turn recognized by RNA polymerase III to form an active initiation "open" complex. Transcription by the enzyme probably results in the denaturation of the template (Sullivan and Folk, 1987), but is not believed to disrupt the complex of TFIIIB, TFIIIC and the DNA (Wolffe et al., 1986). For the following discussion, the definition of a stable complex is a template containing stably bound TFIIIB and TFIIIC. Following the termination of transcription, RNA polymerase III is released from the template and reassociates again with a transcription factor complex to reinitiate transcription (Setzer and Brown, 1985). A number of experiments were carried out to shed some light on the mechanism of 5'-flanking TNNCT function during transcription which would in turn allow the formulation of a model to explain the role of TNNCT in template activity.

A. Transcription competition

The pV4a.5-138,-38G,-35A template was used in a transcription competition experiment with pS7a.5-119, the $tRNA^{Ser}_{7}$ gene subcloned from pDt5. Competition experiments have been used to demonstrate the ability of various templates in forming stable transcription complexes by assaying their ability to affect transcription of a reference gene (Schaack et al., 1983; St. Louis and Spiegelman, 1985; Lofquist and Sharp, 1986). Transcription for pV4a.5-138 wild type and 5'-flanking TNNCT mutant was carried out with the simultaneous addition of pS7a.5-119. As can be seen from Figure 30-A and 30-B, the level of $tRNA^{Ser}_{7}$ gene transcription was reduced more in the presence of the TNNCT mutant than with the wild type template. Therefore the TNNCT mutant was a better competitor for pS7a.5-119 transcription than pV4a.5-138 wild type and did not appear to be deficient in the formation of stable complexes. Hence the lowered Vmax of 5'-flanking TNNCT mutant when compared to its parental wild type, cannot be due to a lesser number of stable complexes formed as a result of the slow formation of the complex (due to reduced binding of transcription factors) or the instability of a formed complex.

It is possible that following complex formation on the $tRNA^{Val}_{4}$ gene, the RNA polymerase III was bound to the complex on the mutant template, but due to the mutation in the TNNCT was unable to initiate as frequently as the wild

type template. In such an instance the polymerase would be less available for binding and directing transcription on the pS7a.5-119 complex. The cycling of RNA polymerase III has previously been demonstrated for transcription of 5S RNA genes (Setzer and Brown, 1985).

In a separate competition experiment aimed at assessing the rate of complex formation for mutant and wild type pV4a.5-138, complexes were preformed by incubation of a constant amount of template DNA with S-100 extract for 0 to 50 minutes. To the preformed complexes was added pS7a.5-119 DNA and the effects on transcription efficiencies of the templates was determined. As shown in Figure 31-B, the transcription complexes on the pV4a.5-138 wild type and mutant templates appeared to be equally stable. Results from the pS7a.5-119 template (Figure 31-C) showed that the $tRNA^{Ser}_{7}$ transcription was equally affected by both the $tRNA^{Val}_{4}$ wild type and 5'-flanking TNNCT mutant templates. Therefore complexes on pV4a.5-138 wild type and mutant templates were formed in similar fashion and were not affected by mutations in the 5'-flanking TNNCT.

B. Effect of NaCl

Previous studies on the binding of transcription factors to a yeast $tRNA^{Leu}_{3}$ gene had shown that NaCl affected the efficiency of transcription through the binding of transcription factors. The binding of TFIIIB was found to be more sensitive to NaCl concentration of 140mM than binding of TFIIIC to the T control region (Stillman et al.,

1984a). Since one possible effect of the TNNCT would be to change the binding of transcription factors, the wild type and mutant pV4a.5-138 templates were tested to determine whether they responded differently to the effects of NaCl during transcription.

In one set of experiments, four different concentrations of wild type and mutant pV4a.5-138 were incubated with the S-100 extract for 30 min to form stable complexes and reactions made 140 mM by the addition of NaCl. Following a 60 min transcription, products were analyzed (Figures 32 A and B) and Vmax values derived for the template. The addition of NaCl to the reactions resulted in an approximately 80% decrease in Vmax for both wild type and 5'-flanking TNNCT mutant templates, indicating similar susceptibility of their complexes to the effects of 140 mM NaCl. Although for the yeast tRNA gene reported by Stillman et al. (1984a) 140 mM NaCl primarily affected the binding of TFIIIB, it would appear that this salt concentration may have disrupted binding of both TFIIIB and TFIIIC to the $tRNA^{Val}_4$ genes, thereby severely reducing the level of transcription. Since it was possible that factors may have gradually dissociated from the templates in the presence of 140 mM NaCl, an experiment was carried out to test the effect of 140 mM NaCl on preformed complexes with time.

The time course of the effect of 140 mM NaCl during the course of transcriptions of pV4a.5-138 wild type and mutant templates was also analyzed. Complexes were allowed to form

on a constant input of mutant and wild type template DNA for one hour and reactions made 140 mM by the addition of NaCl. At various times following the addition of NaCl, transcription was terminated and the products analyzed. Figure 33-C shows a graph of data obtained in this experiment. Results showed that the NaCl concentration severely inhibited the transcription of both wild type and mutant templates within ten minutes of its addition to the reactions. Therefore preformed complexes of both wild type and the template mutant in 5'-flanking TNNCT did not appear to be different with respect to the effects of 140 mM NaCl.

Since it was possible that the rather high salt concentration of 140 mM masked any differences in sensitivity to NaCl concentrations between the wild type and mutant pV4a.5-138 templates, various NaCl concentrations were tested for their effects on transcription. Stable transcription complexes of wild type and mutant pV4a.5-138 were formed as before and NaCl added to the reactions at different concentrations. Figure 34-B shows the effect of different NaCl concentrations on preformed complexes pV4a.5-138 wild type and mutant templates. It can be seen from the Figure that both templates were equally affected by the different NaCl concentrations, suggesting the existence of complexes having similar stabilities on both the wild type and mutant templates. In addition, while DNase I footprint data by Stillman et al. (1984a) showed stable TFIIIB association at an NaCl concentration of 120 mM, the results

reported here show that transcription was affected at NaCl concentrations below 120 mM (30 mM and above) for both wild type and mutant templates. Therefore if the binding of TFIIIB from Drosophila was to occur with the same affinity to the yeast tRNA gene, then some other component must be affected in the $tRNA^{Val}_4$ genes, due to the higher sensitivity of the Drosophila tRNA genes to ionic strength.

C. Effect of temperature

Previous studies on the binding of transcription factors to the yeast $tRNA^{Leu}_3$ gene had shown that temperatures in the range of 0 to 20° C affected the binding of factors to the tRNA gene with the most stable interaction occurring at 20° C (Stillman et al., 1985a). The stability of binding was analyzed by footprint analysis and the data showed that a greater amount of binding occurred by increasing the temperature to 20° C. Similar results were obtained by Andrews et al. (1984) and Ruet et al. (1984). In addition, Schaack et al. (1983) showed that while stable complex formation on pArg occurred within five minutes and was independent of temperature between 24 and 30° C, the initiation step which followed was affected by temperatures in the same range. Two experiments were carried out to determine the influence of increased temperatures on the transcriptions of stable complexes formed on pV4a.5-138 wild type and 5'-flanking TNNCT mutant templates.

First, stable complexes were formed at 23.5° C for one hour and subsequently transferred to higher temperatures

where transcription was continued for a further hour. Figure 35-B summarizes the results obtained from this experiment. As seen from the graph, there was an increase in the percent transcription of both wild type and mutant complexes at 28° C, but transcription rate decreased with increasing temperature to 34° C and severely diminished at 37° C. It is important to note that both wild type and mutant templates followed a similar pattern for the effect of increased temperatures. The initial increase in transcription rates due to a shift to 28° C may simply reflect increased polymerizing activity by the polymerase enzyme. However, the effects observed at higher temperatures probably involve the destabilization of specific factors.

In the second experiment, stable complexes were formed with a DNA input range of pV4a.5-138 wild type and mutant templates at 23.5° C and then transferred to 28° C. Transcription products were analyzed (Figure 36) and Vmax values derived for both templates at 23.5 and 28° C. Results showed that while transcription of both mutant and wild type templates increased, the Vmax for pV4a.5-138 wild type increased by two-fold over the Vmax for the 5'-flanking TNNCT mutant.

D. Time course of transcription

The results from sections IV.B and IV.C investigating the effects of temperature and ionic strength showed that stable complex formation for the $tRNA^{Val}_4$ genes was not

affected by altering the 5'-flanking TNNCT sequence. In addition, the results from the competition experiments showed that the mutation in 5'-flanking TNNCT was probably responsible for affecting the rate of transcription initiation due to the arrest of RNA polymerase III on the mutant template (see section IV.A). To determine whether any differences existed in the time for transcription initiation between the wild type and mutant pV4a.5-138 templates during the course of a transcription reaction, template DNAs were incubated with the S-100 extract under standard conditions and transcriptions were terminated every ten minutes for a total of two hours. Previous studies on the time course of transcription had shown that the reaction is non-linear and is characterized by a lag time preceding the time interval in which transcription becomes linear with time (Rajput et al., 1982; Schaack et al., 1983). Data obtained from the transcriptions are shown in Figure 37-C. Results showed that transcription could be detected within ten minutes following the addition of S-100 extract for both pV4a.5-138 wild type and mutant templates. Therefore no significant difference in lag existed for complex formation and transcription initiation between the wild type and mutant templates. The previous results showed that complexes formed on both mutant and wild type templates were equally stable. In addition, the present experiment showed that the rate of formation of these complexes did not vary between the two templates. Therefore the data suggest that

the altered rate of transcription initiation may be one possibility for the effects observed.

E. Transcription initiation

The purpose of the following experiment was to measure specifically the rate of transcription initiation for full-length transcripts of pV4a.5-138 wild type and 5'-flanking TNNCT mutant templates. Since the initiating nucleotide had previously been identified as -9G by S1 mapping (Sajjadi et al., 1987), transcription in the presence of [γ-^{32}P] GTP would allow the detection of full-length, complete transcripts initiating at this nucleotide, although partial elongation products of initiation would not be detected.

Transcriptions were carried out with both wild type and mutant templates, at six different DNA concentrations, in the presence of either [α-^{32}P] UTP or [γ-^{32}P] GTP. If the 5'-flanking TNNCT mutant was not deficient in transcription initiation, then both wild type and mutant templates would have a similar value for Vmax for transcriptions in the presence of [γ-^{32}P] GTP. Results showed that the template defective in 5'-flanking TNNCT was defective in the initiation of transcription and had a Vmax 34% lower than pV4a.5-138 wild type. The drop in Vmax for transcriptions in the presence of [α-^{32}P] UTP was 39% and hence very similar to that observed with [γ-^{32}P] GTP. Though only transcripts longer than ten nucleotides could be detected on these gels, no accumulation of short or incomplete transcripts was detected for either the mutant or wild type

templates. It therefore would appear that the 5'-flanking mutations in TNNCT do not alter the ratio of start to complete transcripts, indicating that the mutations do not affect the elongation reaction. Hence it would appear that TNNCT is responsible for determining the efficiency of the specific initiation of transcription of the $tRNA^{Val}_4$ gene.

V. Modulation of transcription by 5'-flanking sequences of a $tRNA^{Ser}_7$ gene

The $tRNA^{Ser}_7$ gene from pDt5 (Table 4) directs transcription at an efficiency comparable to pV4a.5-179. However, this gene does not contain a TNNCT in its 5'-flank (Figure 24). Since the presence of TNNCT in the 5'-flank was strongly correlated with efficient transcription, the $tRNA^{Ser}_7$ gene represented an exception to this rule and became therefore the subject of further study. To delimit the sequences required for directing efficient transcription of the Ser_7 gene, approximately 300 bp of 5'-flank was deleted from a subclone of pDt5 by restriction with HhaI. The HhaI fragment was subjected to digestion with exonuclease BAL-31 and cloned into pEMBL8-. When deleted clones were sequenced in their entirety, it was found that all clones contained varying amounts of 5'-flanking deletions, but all retained 184 bp of 3'-flanking sequence. BAL-31 termination preference sites had previously been encountered in the 5'-flank of pV4a.5-138 (Sajjadi, 1985).

Table 3 shows the transcription efficiencies of some of the deletion derivatives of pS7a.5-125. Deletion end-point pS7a.5-119 was found to direct transcription at wild type levels (pS5#1, data not shown). Further deletion of 5'-flanking sequences to position -31 resulted in a slight increase in Vmax (8%) relative to wild type. However, deletion to position -24 relative to the mature coding sequence resulted in a 57% decrease in the level of Ser_7 transcription. Deletion end-point -24 had resulted in the removal of the sequence $^{-29}$AGTTG^{-25}. Interestingly, the sequence is also present in the 5'-flank of pV4a.5-138, $^{-46}$AGTTG^{-41}. The disruption of this sequence in the 5'-flank of the $tRNA^{Val}_4$ gene had previously been shown to result in a 25% decrease in the level of transcription relative to pV4a.5-49 (Sajjadi et al., 1987). This suggests that AGTTG represents a second positive modulatory element in the 5'-flank of the $tRNA^{Val}_4$ gene. The sequence was also found in the 5'-flank of a $tRNA^{Arg}$ gene, pDt17R at position $^{-40}$AGTTG^{-36}. The pDt17R gene (Table 4) which also contains eight TNNCTs in its 5'-flank is reported to be the most efficient template for transcription which has yet been identified (C. Newton, personal communication), though the contribution of any of the 5'-flanking sequences to its transcription efficiency remains to be determined.

Further deletion of the $tRNA^{Ser}_7$ 5'-flank to position -18 resulted in an 81.5% decrease in Vmax. Therefore no further deletions of the 5'-flank were created. The

relative position of the AGTTG sequence in the 5'-flank of the Arginine, Serine and Valine tRNA genes suggests that unlike TNNCT which appears to require a distance of 30 nucleotides from the gene for efficient modulation of transcription, the AGTTG functions very efficiently by its closer proximity to the mature coding sequence. The results indicate that the $tRNA^{Ser}_{7}$ gene only requires the immediate 30 nucleotides of its 5'-flank to direct efficient transcription. However, it may be possible for the pS7a.5-119 template to direct transcription more efficiently (perhaps comparable to that of pDt17R or pYH48 or pV4a.5-49) if a TNNCT was present at position -35 in its 5'-flank. Therefore the positive modulation of transcription by 5'-flanking sequences appears to be more complicated than simply having a TNNCT present in the 5'-flank. Additional modulatory sequences may still exist which may operate in concert with TNNCT and AGTTG to determine the efficiency of tRNA transcription. The mechanism of function for AGTTG is open to speculation and in addition to the intragenic TNNCT sequence present in the $tRNA^{Val}_{4}$ gene requires further study.

VI. Modulation of pV4a.5-138 transcription

The results of experiments reported in this study suggest that the sequence TNNCT in the 5'-flank of pV4a.5-138 is not involved in either the rate of complex formation (section IV.A), the maintenance of a stable complex

(sections IV.B,C,D) or the rate of elongation of a preformed complex (section IV.E). Although stable complex formation has been previously shown to be dependent on the 5'-flanking regions, of 5S and tRNA genes (Schaack et al., 1984; Johnson-Burke and Soll, 1985; Taylor and Segall, 1985), it does not appear that TNNCT affects complex formation, although the role of other sequences in the 5'-flank of the $tRNA^{Val}_4$ gene in influencing complex formation cannot be ruled out. In addition, the 5'-flank of the $tRNA^{Val}_4$ gene is not capable of binding a transcription factor required for efficient transcription, at least in the absence of a tRNA gene (section II).

As discussed earlier, the current model for transcription of tRNA genes involves the formation of stable complexes by binding of factor TFIIIC and TFIIIB which are recognized by RNA polymerase III. Stable transcription complexes are formed by the interaction of TFIIIC with the T control region as a first step in complex formation to form a metastable complex (Lassar et al., 1983; Johnson-Burke et al., 1985). The binding of TFIIIB to the metastable complex serves to stabilize this association (Sharp et al., 1985). The tRNA gene containing a stable "preinitiation" transcription complex is then recognized by RNA polymerase III to form an "open" complex which would in turn initiate transcription upstream of the factor binding sites. The role of TNNCT in the model assumes that following the association of polymerase with the stable complex, the

polymerase would also contact the modulatory sequences (such as TNNCT or AGTTG) present in the 5'-flank of the gene. The direct interaction of RNA polymerase III with the stable complex and the 5'-flanking TNNCT would determine the rate of transcription initiation by the bound polymerase. Following one round of transcription on the template, the polymerase would dissociate, leaving TFIIIB and TFIIIC bound on the template. The cycle would then be repeated (Figure 39). Recent studies have shown that following stable complex formation, TFIIIC and TFIIIB remain stably bound to the template prior to reinitiation by RNA polymerase III (Gottesfeld, 1986; Felts et al., 1987).

The 5'-flanking TNNCT of $tRNA^{Val}_4$ gene may be involved in providing a signal during the interaction between the 5'-flank and the polymerase to influence the frequency of transcription initiation. The results of experiments reported in this study have shown that formation of a stable complex was not affected by the mutation in 5'-flanking TNNCT. The lowered transcription rates of the TNNCT mutant suggest that the initiation of transcription has been affected. The results are therefore consistent with a possible role for TNNCT in the step following complex formation in a two-step model for stable complex formation (Sharp et al., 1985; see also section III. in the Introduction). The interaction of RNA polymerase III with the 5'-flanking sequences of tRNA genes has been previously considered by Morton and Sprague (1982) and more recently by

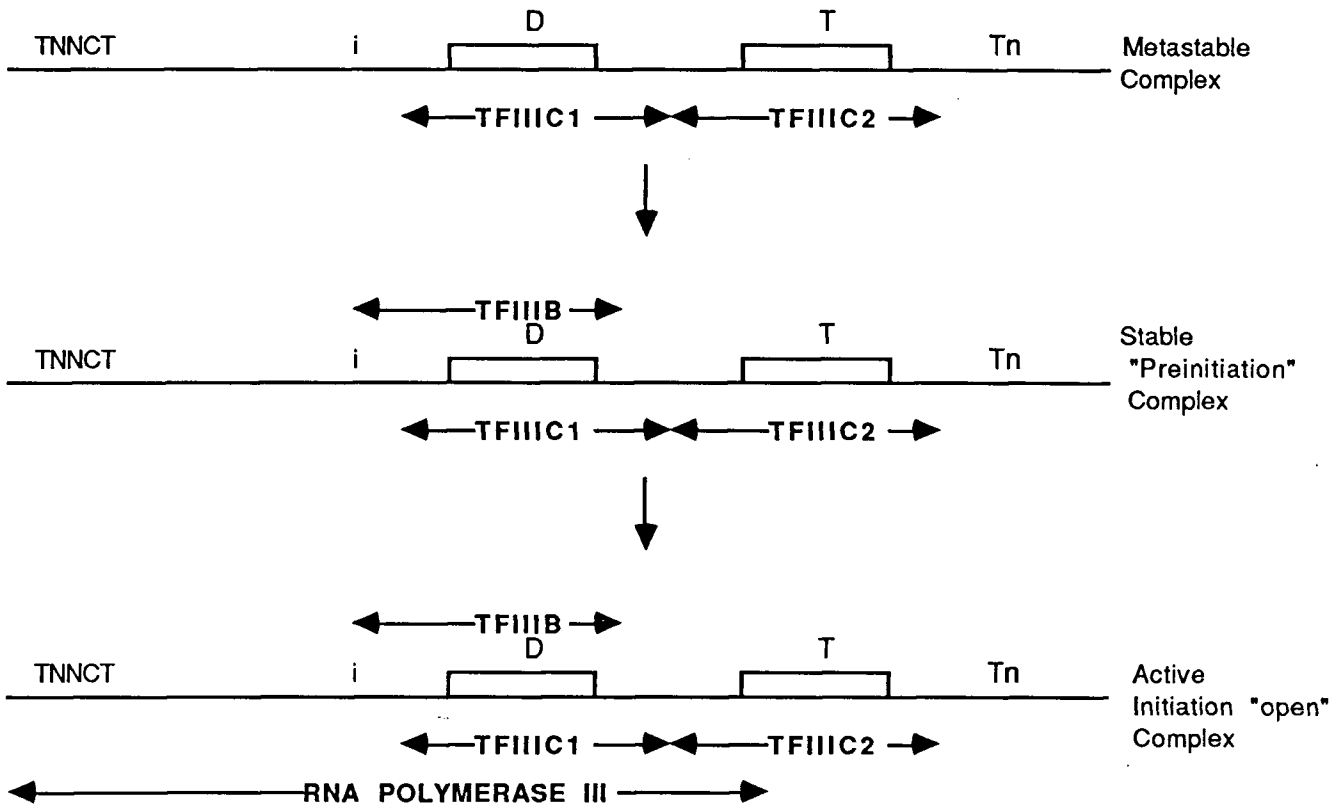
Lofquist and Sharp (1986) and may be eventually demonstrated in a purified transcription system.

The effects observed for the mutation in the intragenic TNNCT are probably unrelated to those observed with the 5'-flanking TNNCT mutant. This suggests that the model for the control of tRNA gene transcription is more complicated, since in addition to the modulatory effects of 5'-flanking sequences on the D and T control regions, additional sequences in the gene also play a role in directing transcription.

The many different control sequences present in the 5'-flanking regions of tRNA genes and the incompatibility of 5'-flanking and intragenic sequences between different tRNA genes (Cooley et al., 1985) reflect the degree of complexity required to bring about transcriptional regulation of the vast number of tRNA genes <u>in vivo</u>.

Figure 39 A model for the mechanism of tRNA gene transcription

The model shows the structure of a tRNA gene and various control sequence elements. Tn, T, D, i and TNNCT correspond to the poly T termination signal, the T-control, and the D-control regions, the region of transcription initiation and the positive modulatory sequence respectively. Arrows define the boundaries of DNA or protein contacts for transcription factors and RNA polymerase III. The pathway to stable complex formation is initiated by the interaction of TFIIIC2 with the T-control region and TFIIIC1 with the D-control region. This metastable complex is then recognized by TFIIIB which serves to stabilize the complex by protein-protein contacts alone. Finally, RNA polymerase III recognizes the stable complex of TFIIIC2, TFIIIC1 and TFIIIB and by binding to the complex, contacts TNNCT in the 5'-flanking region. The rate of transcription initiation is modulated by TNNCT and following termination of transcription, the enzyme dissociates, leaving the stable complex free to reassociate with RNA polymerase III.


TNNCT
i
D
T
Tn
Metastable Complex
TFIIIC1
TFIIIC2
TFIIIB
Stable "Preinitiation" Complex
Active Initiation "open" Complex
RNA POLYMERASE III

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