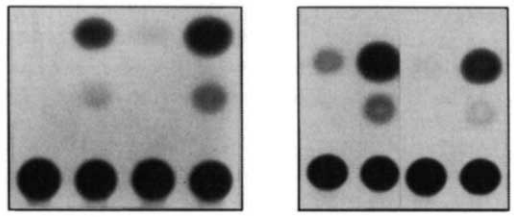

X-linked αCD3								
Cotransfected plasmid	Control		c-*fos*		Ctrl	Fos	Ctrl	Fos
% conversion	0.2	3.1	0.35	11.8	4.5	39.8	2.2	17.7

Murine 3xNFAT/OCT -CAT

FIG. 4 Cotransfection of a c-*fos* expression plasmid augments the induction of the murine 3xNFAT/OCT-CAT plasmid in response to anti-CD3ε stimulation in Ar-5 cells. Ar-5 T cells were cotransfected as described[11,26] with 2.5–5 μg of the 3xNFAT/OCT-CAT plasmid and 2.5 μg of either a human c-*fos* expression plasmid[17] or a control plasmid lacking only the c-*fos* sequences. Transfected cells were activated the next day with the indicated concentrations of anti-CD3ε and collected 24 h after activation. The per cent conversion of ^{14}C-chloramphenicol to its acetylated forms was quantified using a Betagen Betascope. The left and right panels show two independent experiments.

to c-*fos* induction, because expression of c-*fos* in resting T cells does not activate NF-AT even though the pre-existing complex as well as all Jun proteins[11] are expressed.

These results are consistent with a model for NF-AT activation which requires two separate stimulation-dependent steps. One step is CsA-sensitive, and probably invoves modification of the pre-existing NF-AT-binding factor and/or its translocation to the nucleus. The other step is insensitive to CsA, and involves the addition of newly synthesized Fos (and perhaps Jun) proteins to the pre-existing NF-AT-binding factor in the nucleus of stimulated T cells. We note that Fos (and Jun) proteins possess all the properties previously postulated for the newly synthesized subunit of NF-AT[8]: presence in nuclear rather than cytosolic extracts, ubiquitous expression in stimulated cells, and insensitivity to CsA and FK506.

Our results add to the increasing number of cases in which crosstalk between AP-1 and other transcription factors has been reported. Direct interactions between Fos/Jun proteins and steroid hormone receptors have been demonstrated[19–22], as have cooperative interactions between Ets proteins and AP-1 (ref. 23). In fact, the GGAA binding sequence[24] required for NF-AT binding is very similar to the core Ets binding sequence[24]. Antibodies to an Ets-1-specific peptide and to a peptide shared between Ets-1 and Ets-2 (ref. 25) did not block binding in gel shift assays (data not shown); however, the NF-AT subunit may be related to a different member of the Ets family. Identification of the core NF-AT subunit(s), and elucidation of the mechanisms of NF-AT/AP-1 interaction, will be necessary to understand IL-2 gene induction and the mode of action of CsA and FK506. □

Received 10 October 1991; accepted 17 March 1992.

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ACKNOWLEDGEMENTS. We thank R. Distel for the Jun antiserum; E. Serfling for the 3xNFAT/OCT-CAT plasmid; S. Porcelli for the human T-cell clone 8S1; and J Cambier for the Ets-1 and Ets-1,2-specific antisera. This work was supported by the NIH (to A.R.), a postdoctoral fellowship from the Lady Tata Memorial Trust (to J.J.), a Special Fellow Award from the Leukemia Society of America (to P.G.M.), and a USDA training grant in Biotechnology to V.V.-A.).

Direct homeodomain–DNA interaction in the autoregulation of the *fushi tarazu* gene

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A MAJOR problem in the elucidation of the molecular mechanisms governing development is the distinction between direct and indirect regulatory interactions among developmental control genes[1–5]. *In vivo* studies have indicated that the *Drosophila* segmentation gene *fushi tarazu* (*ftz*) directly or indirectly autoregulates its expression[6]. Here we describe a generally applicable experimental approach which establishes a direct *in vivo* interaction of the homeodomain protein ftz with the *ftz cis*-autoregulatory control region. *In vitro* studies have shown that the DNA-binding specificity of the ftz homeodomain can be changed by a single amino-acid substitution in the recognition helix (Gln 50 → Lys)[7]. Whereas wild-type ftz homeodomain binds preferentially to a CCATTA motif, the mutant homeodomain (ftzQ50K) recognizes a GGATTA motif. We now find that the *in vivo* activity of an *ftz* autoregulatory enhancer element is reduced by mutations of putative ftz-binding sites to GGATTA. This down-regulatory effect is specifically suppressed *in vivo* by the DNA-binding specificity mutant *ftzQ50K*. These results establish a direct positive autoregulatory feedback mechanism in the regulation of this homeobox gene.

Previous studies have identified a large autoregulatory control region in the *ftz* gene[6,8]. This upstream region contains the 430-base-pair (bp) regulatory element AE (Fig. 1*a*) which directs *lacZ* reporter gene expression in seven ftz-like stripes in transgenic embryos (Fig. 1*b*). This expression is dependent on the *ftz* gene product itself, demonstrating that AE is a direct or indirect target for *ftz* autoregulation (Fig. 1*c*). We have previously identified one high- and five medium-affinity ftz *in vitro* binding sites[8] in AE (termed A1–3, B, C and D in Fig. 1*a*). To test their *in vivo* role, binding sites were deleted in different combinations and *lacZ* reporter gene expression directed by mutant AEs was monitored in transgenic embryos (Fig. 2). Whereas single deletions that remove binding sites A1–3 (transgene AEΔA; Fig. 2*a*) or binding site D (AEΔD; Fig. 2*d*) have no or only a weak effect on enhancer activity, deletions of site B (AEΔB; Fig. 2*b*) or site C (AEΔC; Fig. 2*c*) clearly reduce reporter gene expression. The double deletion constructs removing sites A1–3 and D (AEΔAD; Fig. 2*e*) or A1–3 and C (AEΔAC; Fig. 2*f*) significantly reduce enhancer activity and the double deletion of C and D (AEΔCD; Fig. 2*g*) leads to a very great reduction of reporter gene expression. Triple deletions removing A, C and D (AEΔACD; Fig. 2*h*) reduce enhancer activity to

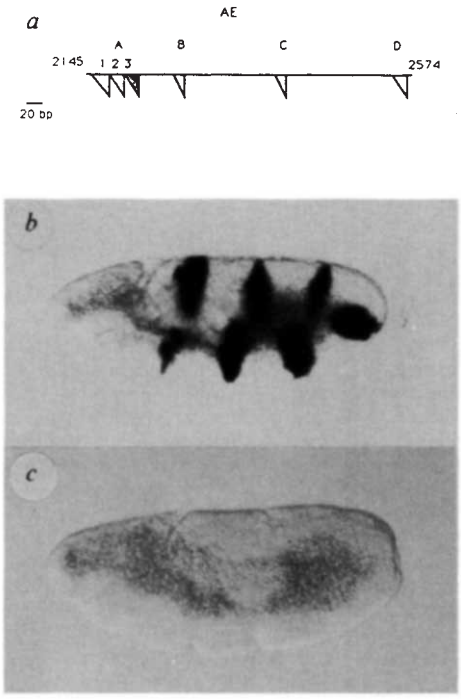

FIG. 1 Expression directed by the 430-bp *ftz* autoregulatory element AE. *a*, The AE region encompasses base pairs 2,145–2,574 (numbering according to ref. 25) in the upstream element[22] and lies 3.5 kb upstream of the *ftz* transcriptional start site. AE contains most or all of the spatial information provided by the previously defined proximal autoregulatory element (ref. 8; A.F.S. and W.J.G., unpublished results) and includes all the sequences that are conserved between the proximal element of *D. melanogaster* and the proximal section of the *D. hydei* autoregulatory region[26]. The AE contains five medium-affinity ftz *in vitro* binding sites (triangles A1, A2, B, C, D) and one high-affinity ftz *in vitro* binding site (triangle A3, ref. 8). It is not known if factors other than ftz protein bind to the regions encompassing the ftz *in vitro* binding sites. The AE and all subsequent derivatives were subcloned in forward orientation into the *hsp70–lacZ* reporter gene vector HZ50PL (ref. 6). This and all other fusion genes were introduced into the germ line of *Drosophila* by P-element-mediated transformation[27]. Transformant embryos were stained for β-galactosidase activity by using the chromogenic substrate X-gal. Expression directed by AE was monitored in wild-type embryos (*b*) and embryos mutant for the ftz amorphic allele ftz^{9H34} (*c*). Anterior is to the left and dorsal is up in all figures.
METHODS. The region encompassing AE (bp 2,145–2,574) was amplified by polymerase chain reaction (PCR)[28] with the internal (sequences present in the ftz autoregulatory region) oligonucleotide primer ASPE23 (5′-CATTTCATCTAGATTACGGGGTCATC-3′) and an external (sequences present in the polylinker) oligonucleotide primer. The amplified fragment and all subsequent derivatives were gel-isolated, digested with *Xba*1 and subcloned into the unique *Xba*1 site of the reporter gene vector HZ50PL[6]. Orientation and sequences were verified by dideoxy sequencing. P-element-mediated transformation, establishment of balanced or homozygous transformant stocks, and detection of β-galactosidase activity was as described previously[6,27]. For AE and all its derivatives, at least five independent lines were established and analysed. Transgenic embryos were stained for 48 h at 37 °C as described[6].

very low levels. Finally, removing all medium- and high-affinity binding sites (AEΔABCD; Fig. 2*i*) abolishes the activity of AE. These results indicate that the regions encompassing ftz *in vitro* binding sites are required for AE *in vivo* activity. All the sites seem to contribute to enhancer activity, although to different extents, and no single site is strictly required, suggesting that ftz-binding sites are combined in a partially redundant manner. This interpretation predicts that the ftz-binding sites are to some extent interchangeable. To test this model, binding site D was replaced with binding site A3 in the context of construct AE–ΔC. As expected for a partially redundant system, the resulting fusion gene AE–A3 is strongly expressed (Fig. 3*c*).

Although the deletion analysis provides evidence that ftz might directly interact with AE *in vivo*, ftz might activate another gene whose product then binds to AE and mediates autoregulation. Other homeodomain proteins expressed during early embryogenesis bind *in vitro* to sites very similar to the high-affinity binding site A3 (refs 9–11). Therefore, it is conceivable that ftz activates another homeobox gene whose product binds to and acts through the putative ftz-binding sites in AE. A powerful genetic approach to identify directly interacting components is the isolation of compensatory second-site suppressor mutations[12]. In this type of analysis a mutation affecting a given component is specifically suppressed by a mutation that causes a compensatory change in a second component with which the first physically interacts (see for example, refs 13 and 14). To find out if ftz protein binds directly to AE *in vivo*, we made use of results from structural and genetic studies on the interaction of homeodomains with their DNA binding sites[7,15–20]. These investigations have shown that amino acid 50 of the homeodomain (amino acid 9 in the recognition helix) is an important determinant of DNA-binding specificity. This residue contacts base pairs 5′ of the ATTA core, which is common to many homeodomain binding sites. In the case of ftz, wild-type ftz

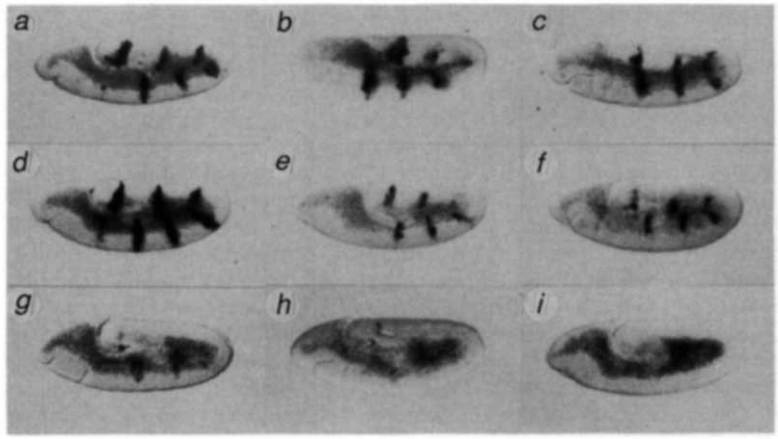

FIG. 2 Deletion analysis of the ftz *in vitro* binding sites in AE. AE fragments missing different ftz *in vitro* binding sites were constructed and expression of fusion genes was monitored in transgenic embryos. Expression of fusion genes: *a*, AE-ΔA (which contains sequences 2,224–2,574); *b*, AE-ΔB (2,145–2,265 and 2,276–2,574); *c*, AE-ΔC (2,145–2,393 and 2,418–2,574); *d*, AE-ΔD (2,145–2,542); *e*, AE-ΔAD (2,224–2,542); *f*, AE-ΔAC (2,224–2,393 and 2,418–2,574); *g*, AE-ΔCD (2,145–2,393 and 2,418–2,542); *h*, AE-ΔACD (2,224–2,393 and 2,418–2,542); and *i*, AE-ΔABCD (2,224–2,265, 2,276–2,393 and 2,418–2,542). No expression of AE-ΔABCD is detectable in four out of six transgenic lines.
METHODS. All deletion derivatives of AE were generated by PCR using previously described strategies[29]. To create internal deletions, two inner oligonucleotide primers introducing deletions were used in two separate PCR reactions extending in opposite directions from the site of the deletion. The gel-purified fragments of the first sets of reactions were annealed and used for a second round of PCR with outer primers. (For detailed protocols and sequences of oligonucleotides, see Supplementary Information.)

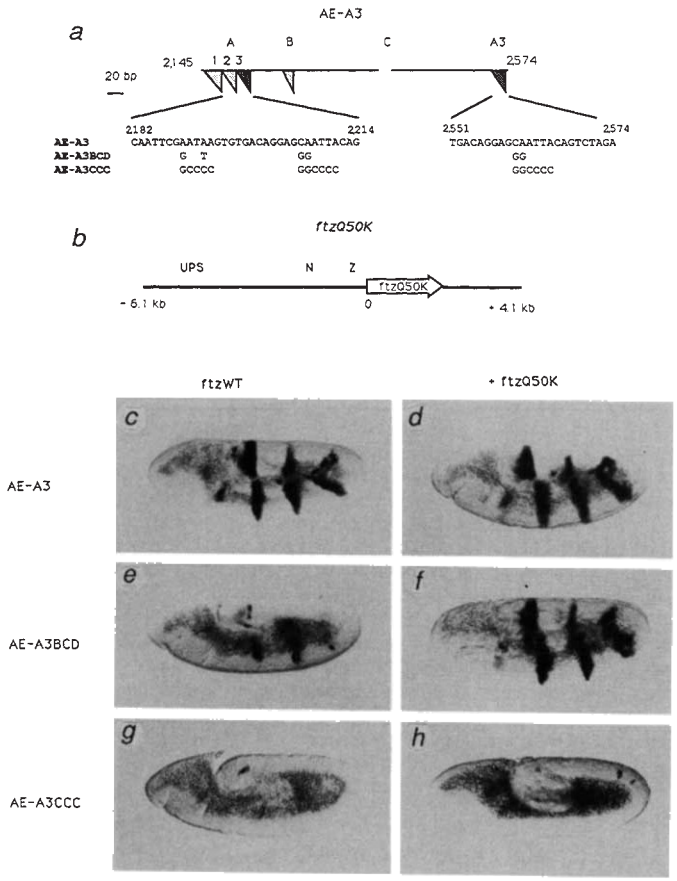

FIG. 3 A DNA-binding specificity mutant of *ftz* suppresses the down-regulatory effects caused by corresponding mutations of ftz *in vitro* binding sites in AE. *a*, Structure of the AE-A3 element and its derivatives. Ftz *in vitro* binding site D in AE-ΔC was replaced with binding site A3. Ftz *in vitro* binding sites A2 and A3 in AE-A3 were mutated to bcd-binding sites giving rise to AE-A3BCD. The ATTA core of the bcd-binding sites was mutated further to CCCC, giving rise to AE-A3CCC. AE-A3BCD and AE-A3CCC also miss ftz *in vitro* binding site A1. Mobility shift assays reveal that the ftzWT homeodomain binds with high affinity to the A3-binding site and with a roughly 10-fold and 100-fold reduced affinity to site A3BCD and site A3CCC, respectively. By contrast, ftzQ50K homeodomain and bcd homeodomain bind with high affinity to A3BCD and with at least 10 times lower affinity to A3 and A3CCC (data not shown). *b*, Structure of the mutant *ftz* gene *ftzQ50K* that carries a single base pair change converting amino acid 50 in the homeodomain from Gln to Lys. This transgene carries all the necessary regulatory elements to direct the expression of ftzQ50K in the domains of ftzWT. UPS, upstream element; N, neurogenic element; Z, zebra element[22]. Expression directed by the different AE-A3 derivatives was monitored in wild-type embryos (*c*, *e*, *g*) or in wild-type embryos containing in addition one copy of the *ftzQ50K* transgene (*d*, *f*, *h*). *c*, *d*, Expression directed by AE-A3; *e*, *f*, expression directed by AE-A3BCD; *g*, *h*, expression directed by AE-A3CCC. Although there are three high-affinity bicoid binding sites in fusion gene AE-A3BCD, no expression is detectable in the anterior region of transgenic embryos, where bcd protein is present at high levels during early embryogenesis (*e*). This agrees with a previous study that reported that a regulatory element that contains five bcd-binding sites failed to direct expression of the *hsp 70-lacZ* reporter gene used in our study[30]. When joined to the basal promoter of the *Krüppel* gene, the same element was able to direct gene expression in the early embryo. These findings suggest that the ability of bcd-binding sites to act in an enhancer element is strongly dependent on the reporter gene construct used[21,30]. Expression of AE-A3CCC is comparable to the deletion construct removing sites A, C and D (AEΔACD in Fig. 2*h*), confirming the results of the binding-site deletion analysis. The presented data provides direct *in vivo* evidence for the binding of ftz protein to region A of AE. It is possible that factors other than ftz bind to AE, including the regions encompassing the ftz-binding sites.

METHODS. To generate the different AE-A3 derivatives, we used PCR as described (Fig. 1 legend) and fusion gene AEΔC as a template. In AE-A3 the underlined sequences encompassing region D (2,551 TGAAGATTACTTCATTTATCTAGA 2,574) are replaced with the underlined sequences encompassing region A3 (2,551 TGACAGGAGCAATTACAGTCTAGA 2,574). The mutant *ftz* gene *ftzQ50K* was obtained by site-directed mutagenesis (Amersham's oligonucleotide-directed *in vitro* mutagenesis system version 2). The oligonucleotide Q50KASmrf1 (5'-CATGCGTCGGTTTTTGAACCAGATCTTG-3') was used to mutate the codon CAA encoding Gln at position 50 of the ftz homeodomain to codon AAA encoding Lys. With the exception of some polylinker sequences and the Q50K mutation, the *ftzQ50K* transformation vector (pC20ftzQ50K) is identical to the transformation vector prf20 (corresponding to the P element P(ry,ftzG)) which complements ftz mutations[22]. Detailed cloning protocols and oligonucleotide sequences are available (see Supplementary Information). Four independent transformant lines were established for *ftzQ50K*. Embryos were collected and stained from crosses of flies carrying the different AE-A3 derivatives with flies carrying the *ftzQ50K* transgene.

homeodomain binds *in vitro* with high affinity to a CCATTA (refs 7, 11) or CAATTA motif (ref. 8), but only weakly to a GGATTA motif[7,18]. This motif is present in binding sites for the homeodomain protein bicoid (bcd)[21]. Converting amino acid 50 in the ftz homeodomain from Gln to Lys (as found in bcd) enables the mutant ftz homeodomain (ftzQ50K) to bind with high affinity to the bcd-binding site[7]. To determine if ftz protein interacts directly with the *ftz cis*-control region *in vivo*, we applied these *in vitro* observations, on the basis of the following logic. If wild-type ftz (ftzWT) directly recognizes AE *in vivo*, an AE whose putative ftz-binding sites were mutated to bcd-binding sites would have a reduced affinity for ftzWT and would have reduced enhancer activity *in vivo*. Introduction of a transgene encoding the ftz DNA-binding specificity mutant ftzQ50K into this genetic background could have two possible consequences on the expression of the mutant AE. In the case of a direct autoregulation, ftzQ50K would be able to recognize the mutant AE and act as a dominant, allele-specific, second-site suppressor restoring enhancer activity. Alternatively, in the case of an indirect autoregulation, ftzWT would act through another gene whose product then in turn binds to AE. In this case no suppression would be expected on introduction of ftzQ50K. Specific suppression could only be achieved by changing the DNA-binding specificity of the (unknown) gene product mediating ftz autoregulation through the putative ftz binding sites.

Ftz binding sites A2 and A3 in AE-A3 were mutated to bcd consensus binding sites (AE-A3BCD, Fig. 3*a*). As a negative control, the ATTA core of these sites was further mutated to CCCC (AE-A3CCC, Fig. 3*a*). Expression of AE-A3BCD is reduced (Fig. 3*e*) as compared with the wild-type construct (Fig. 3*c*) but not as severely as expression of AE-A3CCC (Fig. 3*g*). This suggests that ftzWT binds and activates weakly through the bcd-binding sites in AE-A3BCD.

To test for a direct autoregulatory interaction, a single base pair in the 10-kilobase (kb) *ftz* gene[22] was mutated *in vitro* to convert amino acid 50 in the homeodomain from Gln to Lys (transgene *ftzQ50K*, Fig. 3*b*). Transgenic fly lines were established for *ftzQ50K*, crossed to lines carrying the different AE-A3 derivatives and reporter gene expression was analysed in their progeny. As expected for the case of a direct autoregulatory interaction, the presence of ftzQ50K restores enhancer activity of the AE-containing bcd-binding sites (Fig. 3*f*). This suppression is seen neither with an additional copy of ftzWT (data not shown) nor if the bcd-binding sites are mutated (Fig. 3*h*). These *in vivo* results, together with previous genetic and *in vitro* binding studies[6-8], provide compelling evidence that part of the molecular basis of the self-enhancement of *ftz* gene expression is a direct positive autoregulatory feedback loop in which the homeodomain protein ftz binds to and enhances transcription through the *ftz cis*-regulatory region. Note that the finding of direct autoregulation does not exclude the possibility that there might also be an indirect effect in *ftz* autoregulation.

Our *in vivo* data confirm two important observations previously made *in vitro* and in cell culture systems. First, we find

that ftz acts as a DNA-binding transcriptional activator during *Drosophila* embryogenesis[4,5,23,24]. Secondly, our results verify that amino-acid residue 9 in the homeodomain recognition helix is a major determinant of the DNA-binding specificity of homeodomain proteins and contacts bases just 5′ of the ATTA core motif[7,15–20].

The demonstration of direct *ftz* autoregulation was made possible by combining the classic genetic concept of allele-specific, second-site suppressor mutations with the *in vitro* design of DNA-binding specificity mutants. This class of mutants has so far been used to define specific amino acid-base pair interactions in protein–DNA complexes or to identify rules that assign members of a gene family to subfamilies with different target site specificities[7,14–17]. We have now reported the novel use of a DNA-binding specificity mutant as a tool to establish a direct interaction of a transcription factor and a DNA target site in a multicellular organism. This *in vivo* approach can be applied to any system where transgenic organisms can be generated and should help the analysis of the complex regulatory networks governing development. □

Received 28 October 1991; accepted 5 March 1992.

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SUPPLEMENTARY INFORMATION. Requests should be addressed to the London editorial office of *Nature*.

ACKNOWLEDGEMENTS. We thank M. Affolter, K. Cadigan, B. Karobath, M. Müller, L. Pick, C. Wilson and in particular A. Percival-Smith for helpful discussions, M. Affolter, K. Cadigan, M. Mlodzik and A. Percival-Smith for comments on the manuscript, M. Müller and K. Cadigan for homeodomains and plasmids and P. Jenö for oligonucleotide synthesis. Research was supported by the Swiss National Science Foundation and the Kantons of Basel.

In vitro guide RNA/mRNA chimaera formation in *Trypanosoma brucei* RNA editing

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THE post-transcriptional processing of various mitochondrial transcripts in kinetoplastids, kRNA editing, adds and removes uridines, producing mature messenger RNAs[1,2]. This editing seems to be directed by 'guide' RNAs (gRNAs) which are complementary to portions of the mature message[3]. The editing mechanism has been proposed to entail transesterification[4,5]. Detection of chimaeric gRNA–mRNA molecules, intermediates predicted by transesterification, support this model[4]. We report here the *in vitro* formation of such chimaeras where endogenous gRNAs are covalently linked to added synthetic mRNA. Addition of gel-purified gRNAs to the standard reaction mix increases chimaera formation. This increase is not observed when the gRNA 3′-hydroxyl group is chemically modified, identifying this terminal hydroxyl as the reactive group. These results provide the first experimental evidence for an *in vitro* RNA editing event and support the involvement of transesterification as a chemical mechanism.

All but the terminal regions of ATPase 6 (A6) mRNA are extensively edited in *Trypanosoma brucei*[6] and we have identified several gRNAs that could direct this editing. This includes gA6-14 which is complementary to the furthest 3′ edited sites[6]. The 5′ end of gA6-14 is complementary to 12 nucleotides (nt) at the 3′ end of the A6 message which are not edited in the mature mRNA (Fig. 1*a*). This complementary region can form an 'anchor' duplex which has been proposed to initiate the editing process[3]. We constructed a clone, 3′A6UK, such that 100 nt of pre-edited A6 mRNA with 72 nt of vector sequence at its 3′ end is produced on *in vitro* transcription (Fig. 1*b*). These transcripts were incubated in mitochondrial extracts to determine whether endogenous gRNAs would transesterify *in vitro*. Complementary DNAs were then made using reverse transcriptase and BS-KS, an oligonucleotide primer specific for the vector sequence. The resultant cDNAs were amplified by polymerase chain reaction (PCR) using BS-KS and gA6-14c, an oligonucleotide specific for the gA6-14 gRNA.

Chimaeric molecules formed between the added mRNA and gRNAs should yield a PCR product of 116 base pairs (bp) or larger, depending on the site of transesterification. PCR products between 120 and 180 bp were visible when stained with ethidium bromide after two rounds of 30 cycles of PCR amplification. Cloning and sequencing of the PCR products showed that endogenous gA6-14 gRNAs were covalently linked to the synthetic mRNA at a variety of editing sites (Fig. 2). The 35 chimaeric gRNA–mRNA clones yielded 17 different sequences, all with the gRNAs linked in the editing domain of the A6 mRNA. Most of the cDNAs have the gRNA linked in editing sites 1–16, which are specified by gA6-14. Three cDNAs (A6CIV31, 21 and 45) have the gRNA linked at sites 5′ to the region that complements gA6-14 indicating that this gRNA can edit this region[7]. The mRNA downstream of the gRNA is unedited except in A6CIV14 which lacks one of the two uridines that are deleted in mature mRNA. This single case may represent complete *in vitro* editing at this site. Control PCR amplifications using an oligonucleotide specific for gA6-48 (refs 6, 7), an A6 gRNA that cannot form an anchor duplex with the input RNA substrate, did not generate any chimaeric product.

The 3′ region of the gRNA portion of the chimaeric molecules varies by as much as 15 nucleotides. The longest gRNA sequence in the chimaeras extends 3 nucleotides beyond the region of perfect duplex between the gRNA and mRNA. Most of the gRNA sequences were 8–11 nucleotides shorter than the gRNA sequence in Fig. 1. This heterogeneity in the 3′ region of the gRNA is also observed in chimaeras derived from *in vivo* RNA[8]. Only three of the 17 different chimaeric molecules contain non-encoded uridines at the gRNA–mRNA junction. In the transesterification model of editing, uridines are provided by