Supplementary data for

IDENTIFICATION OF MULTIPLE RATE-LIMITING STEPS DURING THE HUMAN MITOCHONDRIAL TRANSCRIPTION CYCLE IN VITRO*

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Running title: Human mitochondrial transcription

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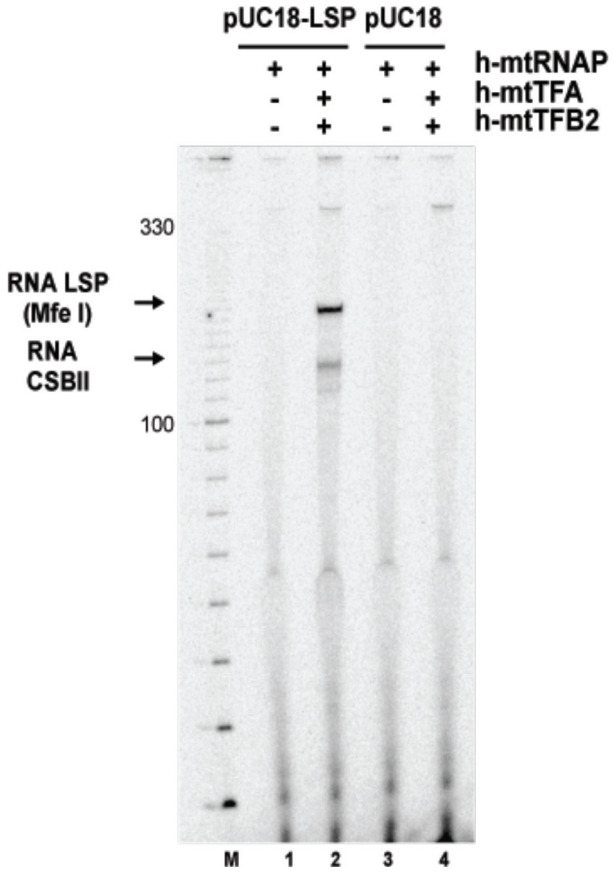

Figure S1. **Specificity of human mitochondrial transcription machinery produced in E. coli.** Transcription reactions were performed on pUC18-LSP (lanes 1 and 2) or pUC18 (lanes 3 and 4) in the absence (lanes 1 and 3) or presence of h-mtTFA and h-mtTFB2 (lanes 2 and 4). Production of RNA that is 100- to 300-nt in size requires both LSP and transcription factors as none of these products are observed by using pUC18 as a template. Reactions were performed by combining h-mtTFA (100 nM) and h-mtTFB2 (20 nM) to linearized plasmid DNA (4 nM) in reaction buffer containing NTP mix (400 μM ATP, 150 μM CTP, 150 μM GTP, 10 μM UTP, 0.2 μCi/μL [α-^{32}P]UTP) at 32 ^{o}C, initiated by addition of h-mtRNAP (20 nM) and quenched after 30 min by addition of stop buffer. Products were resolved by denaturing PAGE on a 5% gel and visualized by phosphorimaging. The size of selected bands from a 10-bp DNA ladder (M) is indicated as a reference.

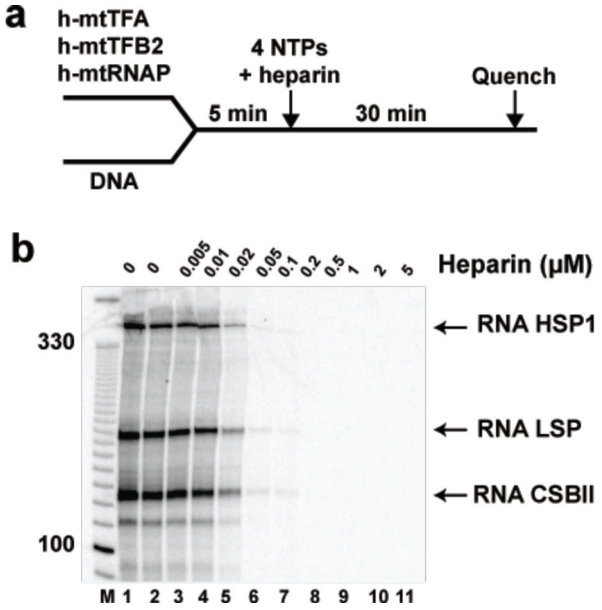

Figure S2. **Heparin inhibits mitochondrial transcription initiation in vitro.** (**a**) Experimental design. Reactions were performed by combining h-mtTFA (100 nM), h-mtTFB2 (20 nM) and h-mtRNAP (20 nM) with linearized pUC18-LSP-HSP1 (4 nM) in reaction buffer at 32 ^{o}C for 5 min, transcription was initiated by addition of NTP mix (400 μM ATP, 150 μM CTP, 150 μM GTP, 10 μM UTP, 0.2 μCi/μL [α-^{32}P]UTP) in the absence or presence of increasing concentrations of heparin (0.005- 5 μM). The reactions were quenched after 30 min by addition of stop buffer. Products were resolved by denaturing PAGE on a 5% gel and visualized by phosphorimaging. The size of selected bands from a 10-bp DNA ladder (M) is indicated as a reference. (**b**) Representative gel showing that transcription on both LSP and HSP1 is inhibited by heparin.

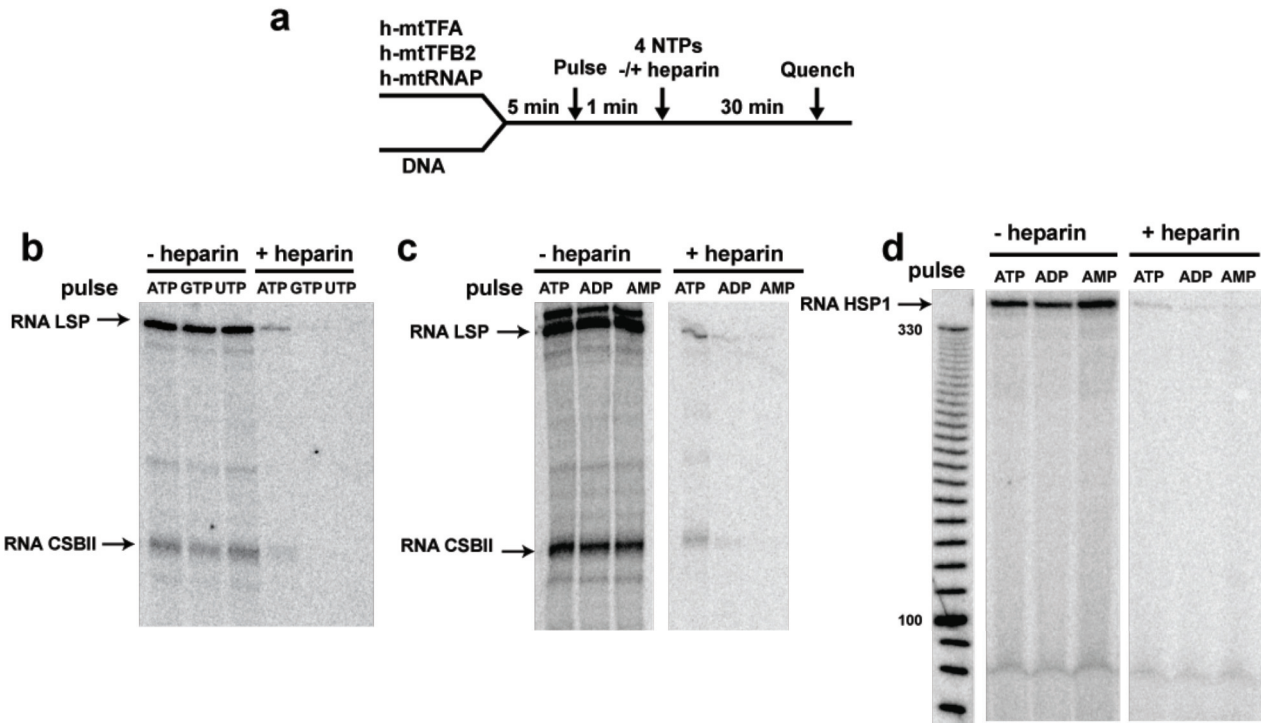

Figure S3. **Requirements for production of heparin-resistant, transcription-initiation complexes.** (**a**) Experimental design. Reactions were performed by combining h-mtTFA (100 nM), h-mtTFB2 (20 nM) and h-mtRNAP (20 nM) with the indicated linearized plasmid DNA (4 nM) in reaction buffer at 32 ^{o}C for 5 min. Reactions were pulsed with the indicated nucleotide (400 μM) for 30 sec prior to initiating transcription elongation with all four NTPs (400 μM ATP, 150 μM CTP, 150 μM GTP, 10 μM UTP, 0.2 μCi/μL [α-^{32}P]-UTP) in the absence or presence of heparin (1 μM). Reactions were quenched after a 30-min incubation. Products were resolved by denaturing PAGE on a 5% gel and visualized by phosphorimaging. The size of selected bands from a 10-bp DNA ladder (M) is indicated as a reference. (**b**) Neither GTP nor UTP (incorrect nucleotides) support formation of heparin-resistant complexes on LSP. (**c**) The yield of heparin-resistant complexes on LSP is reduced by using ADP or AMP instead of ATP. (**d**) The yield of heparin-resistant complexes on HSP1 is reduced by using ADP or AMP instead of ATP.

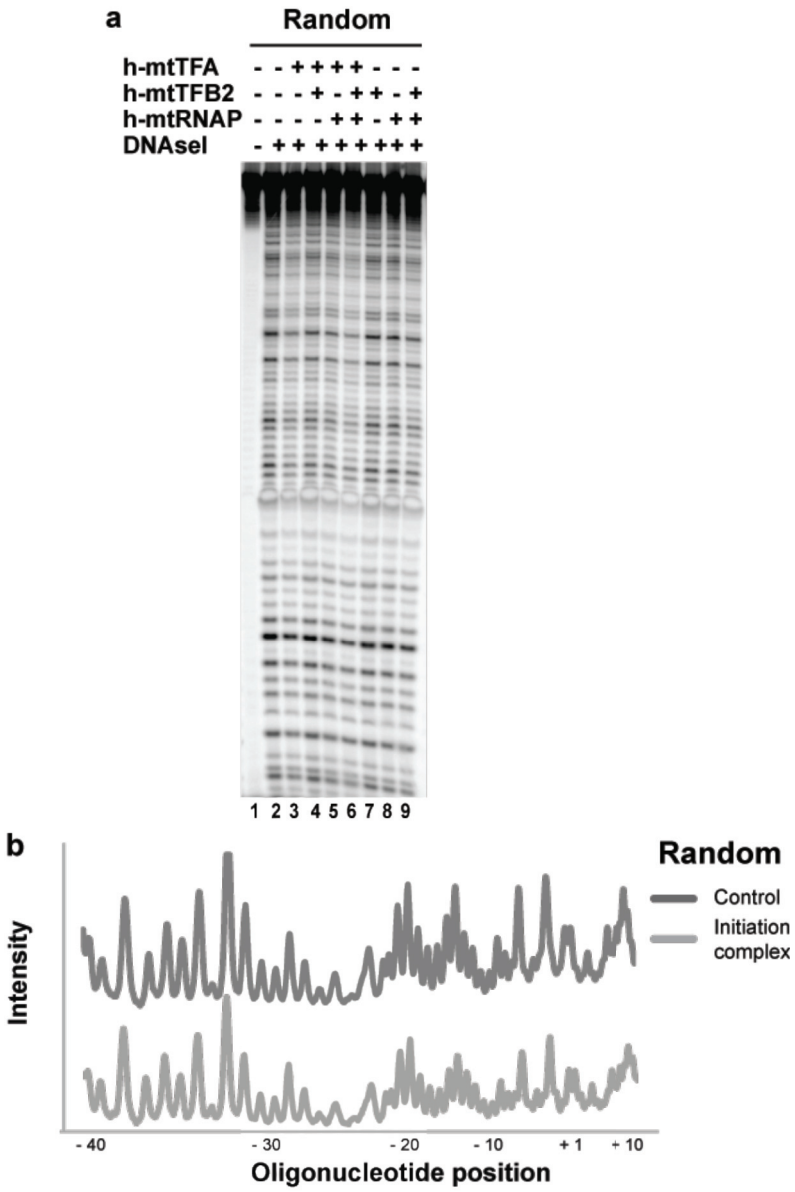

Figure S4. **DNase I footprinting supports specificity of transcription complex assembly on DNA oligonucleotides.** DNase I footprinting was performed using randomized LSP1 (random) ds DNA oligonucleotide templates. Proteins were assembled on the indicated ^{32}P-labeled DNA oligo (2.5 μM) by combining one or more of the following components: h-mtTFA (2.5 μM), h-mtTFB2 (2.5 μM) and h-mtRNAP (2.5 μM), as indicated, in reaction buffer at 32 ^{o}C. DNA cleavage was initiated by addition of RQ1 DNase (0.002 units/μL) and $CaCl_2$ (1 mM) and quenched after 2 min by addition of stop/trap buffer. Products were resolved by denaturing PAGE on 8% gels and visualized by phosphorimaging. (**a**) DNAse I footprint was determined by PAGE. A 10-bp ladder was used as a size marker (M). (**b**). Quantification of selected lanes was performed as described under Experimental Procedures. The data were normalized to the -40 position. The control is from lane 2 and the initiation complex is from lane 6.

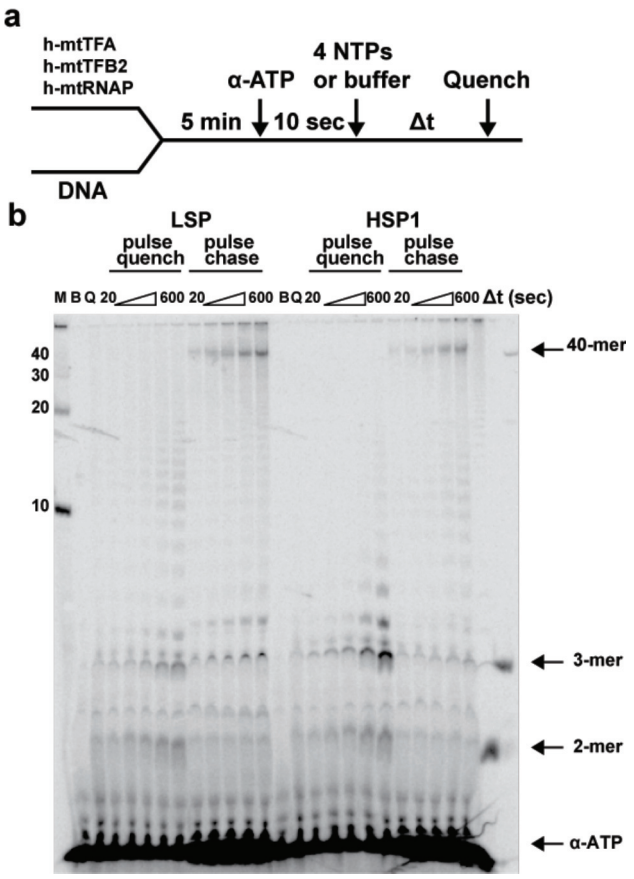

Figure S5. **Di- and trinucleotides formed during initiation can be chased quantitatively into full-length RNA.** (**a**) Experimental design. Reactions were performed by combining h-mtTFA (2.5 μM), h-mtTFB2 (2.5 μM) and h-mtRNAP (2.5 μM) with LSP or HSP1 DNA oligonucleotide template (2.5 μM) in reaction buffer at 32 ^{o}C for 5 min. The assembled complex was pulsed with [α-^{32}P]ATP (100 μM, 0.2 μCi/μL) for 10 seconds, then either buffer (pulse quench) or all four NTPs (500 μM each) (pulse chase) were added. Reactions were quenched at the indicated times. Products were resolved by denaturing PAGE on a 25% gel and visualized by phosphorimaging. The size of selected bands from a 10-bp DNA ladder (M) is indicated as a reference. (**b**) Representative gel. Under pulse-quench conditions (absence of cold NTPs) on LSP and HSP, di-, tri- and tetranucleotides are visible from the beginning of the experiment and continue to accumulate. At later time points, an A-ladder begins to appear. In no case is 40-nt RNA observed. In the chase, di-, tri- and tetranucleotide RNAs produced in the pulse are chased into full-length RNA as labeled di-, tri- and tetranucleotide do not accumulate.

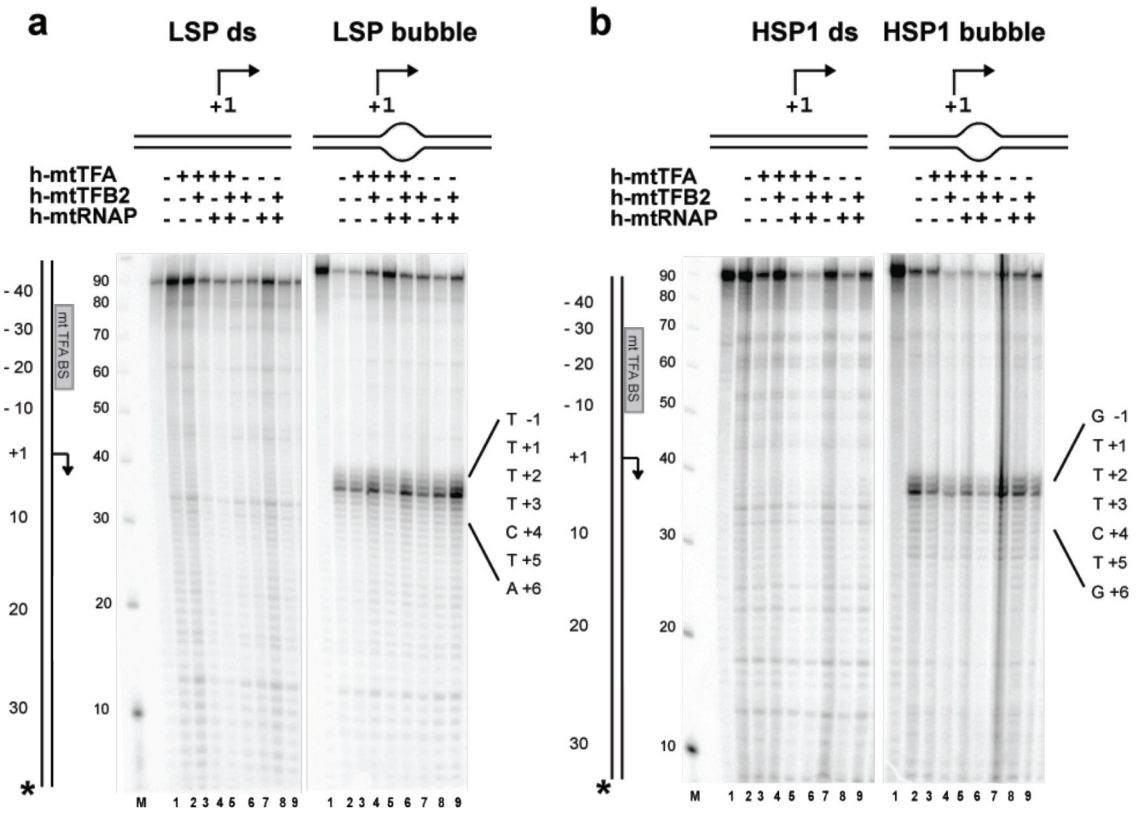

Figure S6. **Open complexes can only be detected on bubble templates by using $KMnO_4$ reactivity.** KMnO4 reactivity was evaluated using LSP, HSP1 or randomized LSP (random) double-stranded (ds) or bubble DNA oligonucleotide templates. Proteins were assembled on the indicated ^{32}P-labeled DNA oligo (2.5 μM) by combining one or more of the following components: h-mtTFA (2.5 μM), h-mtTFB2 (2.5 μM) and h-mtRNAP (2.5 μM), as indicated, in reaction buffer at 32 ^{o}C for 5 min. DNA modification was initiated by addition $KMnO_4$ (30 mM) and allowed to proceed at 32 ^{o}C for 2 min. (**a**) Representative gel for experiments performed on LSP or LSP-bubble templates. (**b**) Representative gel for experiments performed on HSP1 or HSP1-bubble templates. $KMnO_4$ reactivity was not observed on double-stranded templates under any combination of polymerase and factors, suggesting that the equilibrium does not lie in the direction of open complex. $KMnO_4$ reactivity was observed on bubble templates, and the reactivity was not attenuated by the presence of proteins, thus ruling out another trivial explanation for the absence of $KMnO_4$ reactivity with the ds templates.

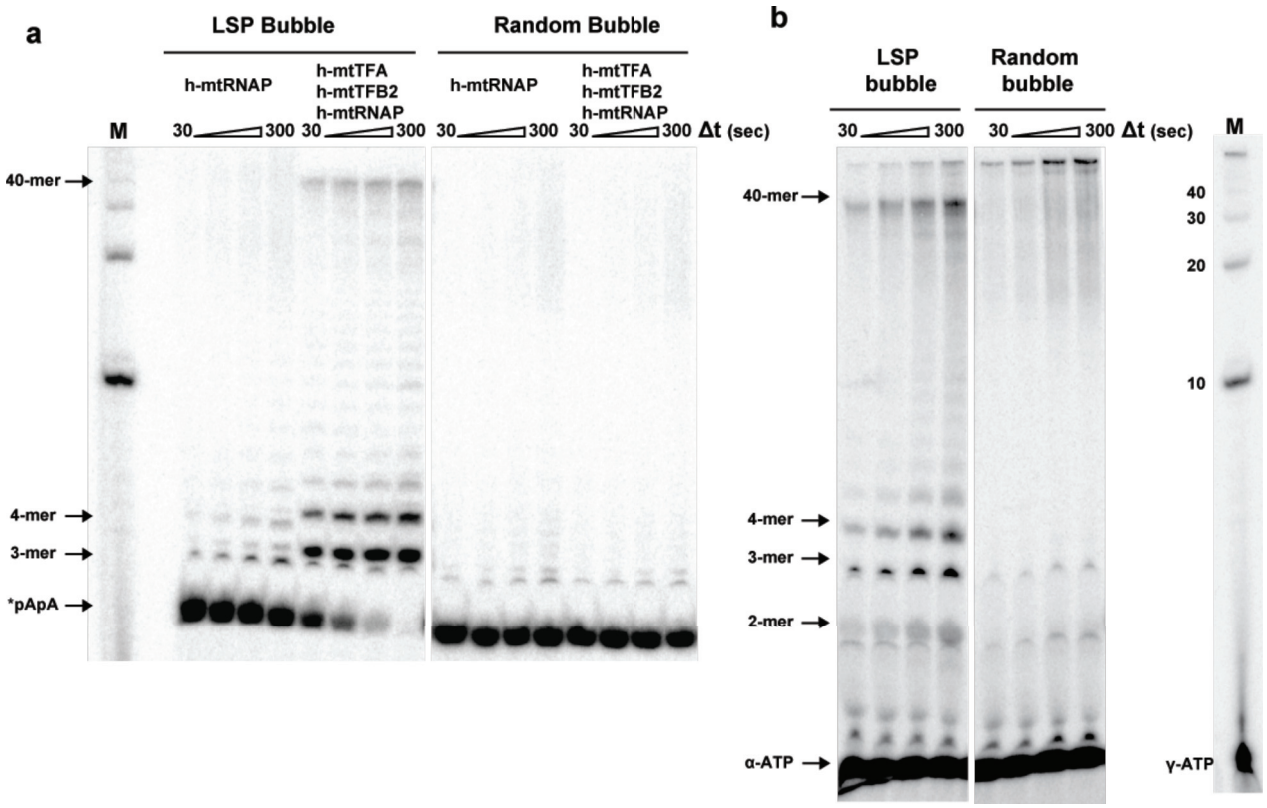

Figure S7. **Bubble templates require a promoter and transcription factors.** Reactions were performed by combining h-mtRNAP (2.5 μM) in the absence or presence of h-mtTFA (2.5 μM) and h-mtTFB2 (2.5 μM) with LSP or random (scrambled version of LSP) bubble templates (2.5 μM) in reaction buffer at 32 ^{o}C for 5 min. The reaction was initiated by addition of NTPs (500 μM each). When ^{32}P-labeled dinucleotide (*pApA) was used, it was added with the proteins at a concentration of 10 μM (final). When α-^{32}P-ATP was used, it was added with the NTPs at the start of the reaction at a concentration of 0.2 μCi/μL (final). Reaction products were resolved by denaturing PAGE on a 25% gel and visualized by phosphorimaging. (**a**) Dinucleotide-primed RNA synthesis on LSP and random bubble templates. Transcription is not observed on random template in the absence or presence of factors. (**b**) De novo RNA synthesis on LSP and random bubble templates. Transcription is not observed on random template in the absence or presence of factors.