DNA repair and antigenic variation in Trypanosoma brucei
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Chapter 4

Search for J-synthesizing enzymes

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Search for J-synthesizing enzymes

The results obtained with nucleoside feeding experiments (van Leeuwen et al. 1998b) and with the hSMUG1 transfectants strongly support the two-step model for J-biosynthesis. A consequence of this model is that there must be a β-glucosyltransferase mediating the conversion of 5-HmU into J. Over the past years, a lot of effort has been made to find this enzyme, without success. The experimental approaches to detect such an activity in trypanosomes were mostly based on the incubation of DNA substrates (DNA of the phage ΦE, containing 5-HmU) with cell lysates of bloodstream form T. brucei (Borst et al., unpublished) in the presence of a sugar donor. The detection method was either radioactive (if $[^{14}C]$ labelled UDP-glucose was used as sugar donor) or by an anti-J antiserum. Both approaches have their disadvantages, however. The first one only works if UDP-glucose is indeed the sugar donor for J-biosynthesis, for which there is no evidence yet. The latter one is complicated by the fact that there is J-containing DNA present in the cell lysate that leads to a considerable background (M.Cross, H.v.Luenen, unpublished). This background problem should be abolished by using procyclic lysates. Insect form trypanosomes do not have J in detectable amounts, but it has been shown that they contain the glycosyltransferase activity (van Leeuwen et al. 1998b), although it is lower than in bloodstream forms.

Materials and Methods

For the in vitro assays oligonucleotides (20-100 pmoles) containing four 5-HmU residues and a biotin at the 5’ end (Sigma) were incubated with a crude cell lysate (ca. 1 μg of total protein) for 30 minutes at 37°C in a final volume of 20 μl of reaction buffer (10 mM HEPES pH 7.9, 5 mM KCl, 2 mM MnCl$_2$, 1 mM MgCl$_2$). In addition, a mix of different sugar donors was added (UDP-, ADP-, CDP-, GDP-, TDG-glucose, UDP-mannose, UDP-galactose, GDP-mannose), each at a final concentration of 5μM. The incubation was stopped by adding NaCl to 1M. Subsequently the reaction mixture was incubated with 5μl of a streptavidin magnetic beads solution (Dynal™) for 1 hour at RT on a rotation wheel. The beads were caught with a magnet, washed twice with TE buffer (containing NaCl at 1M) and transferred to a nitrocellulose membrane. The anti-J antibody assay was performed as described in van Leeuwen et al. (1997). The lysates were prepared as described in Ulbert et al. (2002). For lysate 2 in figure 3 the cells were only lysed by douncing, the mixture was centrifuged at 10000g for 20 minutes (at 4°C) and the supernatant was used for
the band-shift assay. $^{32}$P-nucleotide postlabelling combined with two-dimensional thin layer chromatography was done as described in van Leeuwen et al. (1998a) with minor modifications (see below). Electrophoretic mobility shift assays were done as described in Cross et al. (1999).

**Results and Discussion**

We used a crude cell lysate of insect form trypanosomes to look for a β-glucosyltransferase activity. The lysate alone did not show any reaction with the J-antiserum (not shown). Incubation of the lysate with 5HmU-containing oligonucleotides led to a positive signal. This signal was not dependent on the presence of the sugar donor-mix. Figure 1 shows the results obtained on single stranded oligonucleotides. The J signal was strongest when the reaction was carried out at 37°C, but was absent at 0°C and 65°C, or when the lysate was boiled before use. The maximum signal was reached after 15 minutes. When an oligonucleotide was used that contained dT in place of 5-HmU, a signal was detected too (dT). However, no signal was detectable with an oligonucleotide that consisted only of dC, dG and dA (no dT), indicating that the signal was at least depending on dT. The signal was still present when the reaction mixture was digested with proteinase K and extracted with phenol/chloroform after the incubation and prior to adding of the beads. The finding that the signal was also there without 5-HmU, the precursor of J, was unexpected. Procyclic trypanosomes were only shown to make J when exogenous 5-HmU was incorporated, indicating that they lack a Thy-hydroxylase activity. However, the signal obtained with Thy was usually weaker than the one with 5-HmU, and, in contrast to the 5-HmU-derived signal, it could be further reduced by the addition of the reducing agent DTT (to 2 or 5 mM, not shown). This indicates that an oxidation of some kind was taking place. Thy can be converted to 5-HmU by oxygen radicals (Rusmintratip and Sowers, 2001) and we can not exclude the possibility that this happened to a small extent during (or before) the *in vitro* assay. The resulting 5-HmU might then have served as a substrate for J-synthesis. To get an idea of the amount of J we detected with our antiserum, we compared the signal to defined amounts of J-oligonucleotides. We found that the 5-HmU-oligo showed a signal corresponding to 3.6 pmoles J. Taken into account the number of 5-HmU residues that were spotted on the filter after the incubation (312 pmoles) we conclude that about 1% of the 5-HmU bases were converted into J. The antibody signal also corresponded to 40 ng of bloodstream form DNA. However, a direct comparison is complicated by the possibility that the oligonucleotides on the beads probably have another accessibility to antibodies than total DNA spotted on a filter.
Figure 1. Antibody signals using the anti-J antiserum on DNA oligonucleotides after incubation with a procyclic cell lysate. Incubation was done at various temperatures and for different time spans. The oligos contain either 5-HmU (5-HmU), deoxy-thymidine instead of 5-HmU (dT), or only dC, dG and dA (no dT).

After having detected a signal using the anti-J antibody, we wanted to verify the result with $^{32}$P-nucleotide postlabelling as an independent approach. This method uses nucleases to digest DNA into single nucleotides. On a two-dimensional thin layer chromatography (TLC) all the different components of the DNA substrate can then be visualised. Thereby J can be detected on the nucleotide level (van Leeuwen et al. 1998a). Postlabelling is usually done using isolated total DNA and therefore, the methodology had to be slightly adapted. After incubation with the lysate, the oligonucleotides were phenol/chloroform extracted and cleaned using the streptavidin-beads. Attached to the magnetic beads, they were subsequently treated with nucleases, resulting in the release of free nucleotides. The beads were then caught with the magnet and the supernatant was further processed.

As can be seen in figure 2 (panel C and D) the oligonucleotides were efficiently digested to single nucleotides which could be visualised on the TLC plate, similar to total bloodstream form DNA (panel B). An oligo containing four J-residues resulted in a strong J-specific signal (panel D). However, the 5-HmU oligo treated with the procyclic lysate showed no signal for J (Panel C). Again, we used a J-oligonucleotide as a control. To keep the amount of input substrate constant we “diluted” the J-oligo in a normal oligo with no base modifications and chose the preparation that gave a comparable antibody signal to the 5-HmU samples. This control assay was performed
twice. Once, we could detect a J-specific signal and once we could not (not shown). In none of these assays we detected a signal with the 5-HmU substrate. We conclude from these result that the amount of J that might have been made by the procyclic lysate is at the detection limit for $^{32}$P nucleotide postlabelling, which has been shown previously to be not the most sensitive method to detect low amounts of J (van Leeuwen et al., 1998a). Therefore, another, more sensitive approach is needed to unambiguously answer the question whether there was really J made in our in vitro assays.

As we have shown that 5-HmU is a free intermediate in J biosynthesis (Ulbert et al., 2002) the glycosylating enzyme probably binds this base. We therefore performed electrophoretic mobility shift assays (band shift assays) with trypanosome lysates and 5-HmU-oligonucleotides (Figure 3). Using various T. brucei extracts we identified a shifted band that was specific for 5-HmU (lane H) as it was not present with the control oligo (T instead of 5-HmU, lane T). However, the shifted band was still present when we co-incubated the reaction with an unlabelled 5-HmU-oligo in 3 to 27 times molar excess (Figure 3 B). Although the control oligo and E. coli DNA could not compete out the shifted band, this result strongly argues against a specific binding of a protein in the lysate to 5-HmU.

In summary, we detected a positive J-antibody signal on 5-HmU-containing oligonucleotides after incubation with a procyclic lysate. The signal was both time and temperature dependent, and comparable to very low amounts of J. However, this result still needs to be confirmed by another, independent method. We tried $^{32}$P-nucleotide postlabelling, but failed to detect J in the samples.
that gave the J-antibody signal. As $^{32}$P-nucleotide postlabelling is a rather insensitive approach, other experimental procedures have to be considered.

![Figure 3](image.png)

**Figure 3.** Band shift assay using crude *T. brucei* lysates on DNA oligonucleotides. **A,** Two lysates showing a 5-HmU-specific shift without competitor. **B,** Competition assay. The competitors were in 3x, 9x, 27x molar excess to the probe. Double stranded *E. coli* DNA was in 30x and 100x weight-excess. H is the 5-HmU-oligo, T is the control oligo. Extract 1 is the standard extract used in the J-synthesis assays, extract 2, see text. The arrowhead represents the shifted band.

**References**


